IN VITRO GROWTH AND MOLECULAR CHARACTERIZATION OF VECTOR-BORNE INTRAERYTHROCYTIC PARASITES OF DOMESTIC ANIMALS AND WILDLIFE

A Dissertation

by

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ABSTRACT

Haemogregarines are a group of blood sporozoans that parasitize reptiles, most commonly turtles, or tortoises. Haemogregarine-like inclusions in the red blood cells of a severely underweight alligator snapping turtle *Macrochelys temminckii* Troost in Harlan were examined in this study. The morphology and morphometric data for intraerythrocytic forms found on microscopic examination were similar to *Haemogregarina macrochelysi* n. sp. previously reported in the same species. The 18S ribosomal RNA (*18S rRNA*) gene was cloned and five sequences deposited in the NCBI GenBank® database. All five showed ~96 % identity to *Haemogregarina balli*, *Hepatozoon* sp., and *Hemolivia stellata*. A phylogenetic tree generated from the five sequences aligned with 18S rDNA sequences of other hematozoa and two outgroup species revealed the cloned sequences clustered on their own branch within the *Haemogregarina* spp. clade. There is no genetic data for *H. macrochelysi* n. sp., so it is unclear if the Texas turtle parasite is conspecific with *H. macrochelysi* n. sp.

Babesia spp. are intraerythrocytic protozoans that parasitize mammals. Cultured *Babesia bovis* and *Babesia bigemina,* parasites of cattle, were recovered from liquid nitrogen (LN_2) storage nearly 30 years after cryopreservation. Four cattle were compared as donors of red blood cells (RBC) and serum for microaerophilous stationary phase (MASP) cultures in the recovery of *B. bigemina*. RBC and serum from only one donor supported the growth of *B. bigemina*. Two *B. bigemina* (frozen in 1986 and 1987) and two *B. bovis* (both frozen in 1986) cryostocks were resuscitated from LN_2 storage and all four recovered and thrived in the donor bovine RBC and serum. In the 3rd passage after recovery, *B. bovis* cultures were cryopreserved. Six months later they were successfully recovered from LN_2 using RBC and serum from the same donor. This

study shows that *B. bovis* and *B. bigemina* stored nearly 30 years in LN_2 can be successfully recovered in the MASP system. This study also confirms previous observations that selection of a suitable bovine donor of RBC and serum is critical to the success of the *Babesia* sp. culture.

Two markers, *18S rRNA* gene and rRNA intervening transcribed spacer regions 1 and 2 (ITS), in *B. bovis* and *B. bigemina* from Puerto Rico (PR) cattle and archived culture samples from Mexico (*B. bovis*) and the Virgin Islands (*B. bigemina)* were PCR amplified, cloned and sequenced. In total, 54 18S rDNA and 21 ITS sequences were deposited in GenBank®. The identity scores among the PR *B. bovis* 18S rDNA cloned sequences were 92.3% to 100%, and 97.7% to 99.99% among the archived Mexico *B. bovis.* PR and the Virgin Islands *B. bigemina* 18S rDNA sequence identity scores ranged from 99.1% to 99.98%. The UPMGA cladogram generated from 18S rDNA sequences shows the clear distinction of *B. bovis* and *B. bigemina* (and *B. ovis)*. The PR ITS cloned sequences showed 69.3% to 100% identity among them. In the UPMGA cladogram, the PR sequences fell into seven different groups, except for one outlier that branched separately.

Thirty cloned *msa-2b* sequences, encoding merozoite surface antigen 2b (MSA-2b), were used to further genotype *B. bovis.* The identity scores among the deduced MSA-2b amino acid sequences ranged from 41.2% to 100% for the PR isolates, and from 45.4% to 100% among the archived Mexico *B. bovis*. The UPGMA cladogram based on MSA-2b separated the sequences into two major clades with the PR and archived Mexico *B. bovis* sequences branching into their own groups within the clades. B-cell epitope predictions showed similar topology between the PR and archived Mexico isolates, although some diversity in sequence was noted. This study revealed heterogeneity in 18S rDNA, ITS1-ITS2 and MSA-2b sequences of the Puerto Rico *B. bovis* isolates suggesting possible origins from different geographic regions.

DEDICATION

I dedicate this work:

To the soul of my father Rasool Alhaboubi.

To my beloved mother for her love and support.

To my wife, Raghad, a best partner and friend during this chapter of our life. Thanks for your love, advice, patience and for the unlimited of encouragement.

Finally, to my lovely butterflies, the Texas girls, LEEN & ROSE, my daughters.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

Apicomplexan parasites share several morphological characteristics which place them into the same phylum. The Apicomplexa phylum includes Haematozoa which include the Piroplasmida (piroplasms) and Haemosporidia (haemosporidia), while the Coccidea include the Adeleina (haemogregarines) and Eimeriina (haemococcidia). Both haemogregarines and piroplasms have apparently evolved in their transmitting vectors more than in the vertebrate host because the sexual stages when recombination occurs take place in their vectors (O'Donoghue, 2017). All members of the phylum are parasitic for a variety of hosts, ranging from primitive invertebrates to human. The parasites features are related to their interactions with the host cell, such as replication and further development of their life cycle stages that are necessary for parasitism (Levine, 1971, 1985). The apicomplexans are intracellular pathogens that have the ability to modify the host cell cytoskeleton before and/or after invasion of the host cell. The parasites survive and interact with distinct metabolic pathways such as gene expression patterns, and the structural organization of the cytoskeletal filaments (Haglund and Welch, 2011; Cardoso et al., 2016). The apicomplexans usually have a complex life cycle, as Ixodid ticks are responsible for transmission of certain intraerythrocytic apicomplexan parasites, including the genus *Babesia* (Bock et al., 2004). On the other hand, haemogregarines utilize a wide variety of reptilian hosts and transmission vectors such as leeches and insects (Ball, 1967; Barta et al., 1989).

Historically conventional biological phylogenies were based on physical characteristics, but more recently molecular phylogenies based on genotypic differences, including the encoded

protein sequences, are employed for molecular taxonomy, population genetics, and phylogenetic analyses (Loker and Hofkin, 2015). Molecular phylogenetics are thought to better reflect evolutionary relationships than phenotypic comparisons (Zimmer and Emlen, 2013). Considerable research has been conducted on *Babesia* due to their economic effects on livestock production as well as disease in pet animals; however, Haemogregarines have been poorly studied from the molecular standpoint. Differences in these two important apicomplexans, namely *Babesia* spp. and *Haemogregarina* spp., will be discussed in this review.

Haemogregarina

General description

Within the Apicomplexan parasites, haemogregarines comprise about 400 species of morphologically similar organisms in four different genera: *Hepatozoon*, *Karylysus*, *Cyrilia* and *Haemogregarina* (Levine, 1985). The genus *Haemogregarina* is mainly identified within the erythrocytic cytoplasm of cold-blooded vertebrates. The infection is found in most reptile species with visualization of the gamont stage, which can be helpful for parasite diagnosis, but is not definitive for identification if used alone (Stacy et al., 2011; Wozniak and Telford, 1991; Telford, 2009). The first described species of *Haemogregarina* parasitizing reptiles was *Haemogregarina stepanow* (Danilewskyi 1885). Historically, *Haemogregarina* was thought to be host specific, thus, each parasite found often led to naming a new species. Twenty-nine named *Haemogregarina* species infect turtles (Levine, 1988). Most of these species were recorded based on the morphological description of a few detected life stages in the intermediate turtle host and are lacking a complete life cycle description.

Although modern molecular genetic tools are now accepted in taxonomic and evolutionary studies, even the more recent descriptions of new species lack supporting DNA sequence data. Among *Haemogregarina* species, to date a few species, such as *Haemogregarina balli* and *H. stepanowi*, have been studied phylogenetically using molecular markers (Barta et al., 2012; Dvořáková et al., 2014).

Morphology and life cycle

Although the life cycle of the haemogregarine group is proposed and suspected to be heteroxenous, it is not yet fully elucidated and verified (Desser, 1993; Reichenow, 1910). For example, there have been variable results in experimental transfer of *Haemogregarina* spp. by leeches to the vertebrate host to test their role as vectors in the life cycle of these parasites (Paterson and Desser, 1976; Khan et al., 1980; Barta and Desser, 1986). However, due to limitations in the detection of infection it is not always clear that the host was naïve at the outset of the study, therefore the results of such studies may not be definitive (Ball, 1970; Paterson and Desser, 1976).

The life cycle for *Haemogregarina balli* describes the introduction of infective merozoites from the leeches to the snapping turtles during feeding. The parasite first invades cells of the lung, liver, or spleen, developing as pre-erythrocytic meronts (Siddall and Desser, 1991). This stage undergoes multiple division to produce numerous merozoites that infect erythrocytes and develop into erythrocytic meronts, yielding eight merozoites. These merozoites exit the cell and infect new erythrocytes, producing either gamonts or more meronts. Gametogenesis generates intraerythrocytic microgamonts and macrogamonts in the peripheral circulation, which are then ingested by leeches during feeding on the infected vertebrates. The sexual phase, syzygy, occurs in the intestinal caeca of the leech with the association of

a microgamont with a macrogamont after the breakdown of the erythrocyte. The microgamont undergoes microgametogenesis, producing four microgametes, one of which fertilizes the associated macrogamete. Sporogony occurs resulting in monosporoblastic oocysts with eight sporozoites, which then migrate to the anterior part of the leech where they develop into primary meronts. Each primary meront contains hundreds of merozoites, which move to the proboscis of the leech where they may be introduced to a host during feeding (Paterson and Desser, 1976; Siddall and Desser, 1990, 1991).

The blood parasites of reptiles are diverse and morphologically variable compared to those of mammalian or avian hosts. The prominent nucleus in the reptile erythrocyte may influence the appearance of the parasite within the cell. Thus, studying and identifying the mammalian piroplasms is simpler compared to looking at populations found in reptiles, which are more diverse (Telford et al., 2009). Furthermore, the *Haemogregarine* apicomplexans have fairly unresolved phylogenetic relationships and evolutionary history.

Babesia

General description

The genus *Babesia* is in the family Babesiidae, order Piroplasmida, class Aconoidasida, and phylum Apicomplexa (NCBI Taxonomy Database https://www.ncbi.nlm.nih.gov/taxonomy). *Babesia* spp. are common intraerythrocytic parasites of cattle, sheep, goats, horses and dogs, although some species may be zoonotic (Levine, 1985). The order Piroplasmida is of great importance in both human and veterinary medicine due to the inclusion of a number of pathogenic species. Among such organisms, those in the genus *Babesia* have received significant attention since they can cause disease in humans, agriculturally important animals (such as

bovine babesiosis), companion animals (canine babesiosis) and in wildlife (Bock et al., 2004). For example, *Babesia bovis* and *Theileria equi* are responsible for large economic losses in livestock production and the equine industry, respectively, while *Babesia microti* is responsible for public health concerns (Zahler et al., 2000; Pérez de León et al., 2010, 2012; Cornillot et al., 2012; Salim et al., 2013; Wise et al., 2013).

Morphology and life cycle

Babesia spp. are a group of vertebrate blood cell parasites which can be either piriform, (pear-shaped), round, amoeboid, or rod-shaped in morphology (Levine, 1985). *Babesia* species differ in size ranging from relatively small intraerythrocytic protozoan parasites such as *B. bovis* with merozoites about 1.0-2.1 μm long to those of large species such as *Babesia bigemina* which measure about 3.0-3.4 μm (Friedhoff, 1981). *Babesia* organelles include polar rings, rhoptries, spherical bodies, mitochondria-like structures, and spheroid bodies which are all improtant for parasite invasion into host cells (Scholtyseck et al., 1970; Friedhoff and Scholtyseck, 1977).

Parasite development in the vertbrate host occurs exclusively in the erythrocytes, with the notable exception of *B. microti* in which the sporozoites may invade the lymphocytes of their mammalian host (Moltmann et al., 1983; Mehlhorn and Schein, 1985). *Babesia* spp. are transmitted by tick vectors. *Babesia* infects the vector tick when blood cells containing the gamont stage are ingested. The gamonts develop into Strahlenkörper (ray bodies) in the intestine of the tick, and then form fused pairs and become zygotes (Mehlhorn and Schein, 1985). In *B. bigemina*, Strahlenkörper enlarge and clump together into aggregates. This phase represents a multiplication of the ray bodies to increase the final numbers of gametes in the gut of the tick (Golgh et al., 1998). The zygote develops into a single kinete and the kinetes leave the intestines

and travel through the haemolymph to organs of the tick, in particular the salivary glands and ovaries of the female tick.

Maturation to infective sporozoites is completed in about five days after the infected larval tick attaches to the host. The active attachment of the sporozoite to the surface of the erythrocyte occurs when the parasite apical pole reorients as necessary toward the cell surface to bring apical organelles close to the erythrocyte membrane for attachment. The rhoptries discharge their contents as the erythrocyte membrane invaginates and the sporozoite enters the erythrocyte (Mehlhorn and Schein, 1985; Hines et al., 1995a; Sam-Yellowe, 1996). Interestingly, immunization with *B. bovis* or *B. bigemina* native or recombinant rhoptry-associated proteins reduced parasitemia in infected cattle (Wright et al., 1992; Norimine et al., 2003).

The parasite, having entered erythrocytes, rapidly undergoes differentiation and enlargement. Multiplication occurs in most *Babesia* species leading to a characteristic appearance of paired merozoites inside an erythrocyte (Mehlhorn and Schein, 1985). In some species, *B. microti* for example, four parasites are formed inside the erythrocyte at the same time leading to a tetrad or Maltese cross arrangement developed from a polymorphic, schizont-like parent cell (Rudzinska, 1981).

Babesia pathogenicity and clinical signs

Among the *Babesia* species, *B. bovis* and *B. bigemina* are highly pathogenic blood borne tick-transmitted protozoans that are causative agents of bovine babesiosis (Levine, 1971). The clinical signs of babesiosis include fever, malaise and inappetence, severe anemia, icterus, haemoglobinuria, hypotensive shock, and enlarged spleen, lymph nodes, and liver (Levine, 1985). In the 19th century, *B. bovis* was first described by Babès (1888) in Romania associated with red water fever in cattle (Uilenberg, 2006). *B. bovis* is often considered more virulent than

B. bigemina because the infected erythrocytes can pass the blood-brain barrier causing cerebral babesiosis with ensuing neurological signs. Bovine babesiosis may result in economic loss due to abortions, loss of milk or meat production, loss of draft power, or even death (Bock et al., 2004).

Historically, bovine babesiosis is of importance because of the 1893 landmark discovery in the United States by Theobald Smith and Fred Kilborne that identified *Pyrosoma bigeminum* (later named *Babesia bigemina*), a protozoan parasite, as the causative agent of Texas cattle fever and that the parasite was vectored by ticks. This was the first confirmation of a disease agent transmitted by an arthropod and led the way for the discovery of other arthropod-borne disease agents, such as those causing malaria and yellow fever.

Among the several *Babesia* species shown to infect bovines, *B. bovis* and *B. bigemina* have the highest impact on cattle health globally (Bock et al., 2004). Bovine *Babesia* spp. are transmitted by Ixodidae tick vectors, which infest cattle, or other bovids and ungulates in most of the world's tropical and subtropical countries (Levine 1985; Uilenberg 2006). *Babesia bovis* and *B. bigemina* are transmitted by *Rhiphicephalus* spp. ticks in Asia, Latin America and Africa.

The Cattle-*Rhipicephalus microplus*-*Babesia* complex has been described as the most important agricultural host-arthropod-pathogen complex globally, in which these one-host ticks complete all developmental stages on the same animal (Mehlhorn and Schein, 1985; Ramos et al., 2010; Heekin et al., 2012). Transovarial transmission of *Babesia* occurs in one-host tick vectors. The *Babesia* migrate to the ovaries of the female ticks, resulting in infected eggs. Infected larval ticks emerge and may then transmit *Babesia* to cattle upon feeding. Transovarial transmission likely contributed to bovine *Babesia* spp. successfully spreading throughout the world.

The severity of the clinical signs due to babesiosis depends on the virulence of the *Babesia* strain as well as host age and is inversely related to the speed of recovery and mortality rates (Bowman, 2006). Bovine babesiosis affects adult animals more severely than young, which can lead to enzootic stability in regions where the climate is favorable to the vector tick and cattle. The tick transmits *Babesia* to calves, which become infected but, do not show clinical signs and remain subclinical carriers (Levine, 1985). Innate and adaptive immunity are both involved in *Babesia* infection (Brown et al., 2006). Immunity is passed from immune cows to their calves via the colostrum, protecting them during the first to the second months of life. However, young animals may remain resistant longer than passively transferred antibodies persist, with apparent non-specific immunity lasting until calves are 7 months old (Mahoney and Ross, 1972; Levy et al., 1982). This age-linked resistance is believed to be innate despite the generally less developed innate immunity of the neonatal calve (Johnston, 1998). This can account for the abundance of γδ T cells among the circulating T lymphocytes in ruminants (Hein and Mackay, 1991). Another possible explanation for the resistance to *Babesia* in young calves is the decrease in the pro-inflammatory response that affects disease pathogenicity (Clark and Jacobsen, 1998). The spleen may play a major role in the immunological basis and innate response to a primary *B. bovis* infection in young calves. This may be explained by the early induction of the immune mediators Interleukin-12 (IL-12) and Interferon-γ (IFN-γ) which are associated with protective immunity. In addition, production of nitric oxide (NO) inhibits parasite growth and is secreted by splenic microphages in calves in higher amounts compared to adult animals (Brown et al., 2006; Goff et al., 2010).

Acquired immunity to *B. bovis* and *B. bigemina* develops in animals that survive an initial infection with this parasite, either naturally or after treatment with chemotherapy. The

animal will remain persistently infected, without developing any further clinical disease. This resistance is termed concomitant immunity, which may last for approximately four years and is dependent on the rapid development of memory and effector CD4+ T helper cells, and antibody produced by B cells (Bock and Vos, 2001; Brown et al., 2006; Norimine et al., 2003). Usually the resolution of *Babesia*-related clinical signs and the immune response are associated with the recurrent antigenic exposure experienced by the host due to the continued presence of live parasites of parasitic protozoa has had a great effect on the development of host-pathogen interaction research and has led to a better understanding of the disease potential and the biology of the parasites.

Parasite in vitro *cultivation*

A continuous laboratory supply is important in providing a living stock of organisms that can then be used in studies of host-pathogen interactions, and to produce quantities of parasites as a source of DNA and/or parasite antigens for molecular, phylogenetic, diagnostic and drug screening studies. In addition, cultures may provide attenuated strains and parasitic antigen for vaccine development, as well as antigen for diagnostic testing (Schuster, 2002a; Visvesvara and Garcia, 2002; Suarez and Noh, 2011).

Bass and Johns in 1912 were the first to attempt cultivating *Plasmodium falciparum* and *Plasmodium vivax* which are intraerythrocytic protozoan parasites related to *Babesia* spp. and are the causative agents of malaria. They targeted the asexual reproduction stages (trophozoite) in the host erythrocytes (Schuster, 2002b). The motivation behind this attempt was based on the fact that malaria is a general health concern and there was an urgent need to develop potential vaccine candidates. Since that time, cultivation experiments of the asexual stages of the malaria

life cycle for both animal and human parasites have been widely used (Butcher and Cohen, 1971; Diggs et al., 1971; Trager, 1971; Schuster, 2002b).

Erp et al., 1978 cultured *B. bovis* in bovine erythrocytes following the methods Trager and Jensen (1976) developed to culture human malaria. The cultures were incubated at 37°C in a candle jar using either RPMI-1640 medium or Medium 199 supplemented with bovine serum. Erp et al. (1980) also evaluated *B. bovis* cultivation using the spinner flask method with magnetic stirrers to keep the cultures constantly agitated using Medium 199 supplemented with 50% bovine serum (Erp et al., 1980). Both of these methods allowed short-term cultivation of the parasite.

The above methods required a large culture to maintain the growth of the parasite, but only allowed low parasite numbers. Thus, this limitation led to the development of the more efficient microaerophilous stationary phase (MASP) technique by Levy and Ristic (1980). By allowing the parasites to proliferate in a settled layer of erythrocytes, while being incubated at 37°C to 38°C in an atmosphere of 5% CO_2 and 95% humidified air, higher percentages of parasitized erythrocytes (PPE) were achieved (Levy and Ristic, 1980). The culture medium consisted of Medium 199 (60%) and bovine serum (40%) supplemented with penicillin G and streptomycin. Rodriguez et al. (1983) in an early evaluation of culture conditions reported that a low oxygen environment is the best for initiating *B. bovis* growth, which is influenced by the depth of the culture medium over the settled layer of erythrocytes.

A modified continuous MASP *B. bovis* culture with Medium 199 and normal adult bovine serum (40%) supplemented with glucose, amphotericin B and gentamycin was reported by Goff and Yunker (1988). This method obtained the maximum percent-parasitized erythrocytes (PPE) of *B. bovis* culture. The observed parasitic stages were either trophozoites

(single parasite), dense forms (no visible cytoplasm), or merozoites (pair of pear-shaped bodies). In this study, [^{3}H] hypoxanthine uptake was used to assess parasite growth *in vitro*. There was a correlation with uptake of [^{3}H] hypoxanthine and PPE, with trophozoites exhibiting the greatest uptake. They also assessed the effect of pH and buffers and found that optimal growth occurred at a pH range of 7.31 to 7.39.

Vega et al. (1985a) were able to design optimal culture conditions for *B. bigemina* based on previous work by Timms in 1980. The culture system was maintained in a 24-well plate and included washing of the RBC before use in culture. The culture medium consisted of Medium 199 with 20-50% normal bovine serum and 5-10% (v/v) normal erythrocytes. Incubation was at 37°C in low oxygen conditions of 2% O_2, 5% CO_2, and 93%N_2 (Vega et al., 1985a).

Developments in biotechnology have led to the introduction of new medium types to be used in cell culture. Using a chemically defined medium, HL-1, which contains transferrin, saturated and unsaturated fatty acids, and insulin (Ventrex Laboratories, Inc., Portland, Maine), supplemented with 20% normal adult horse serum, 2 mM L-glutamine, amphotericin B, and penicillin/streptomycin, Holman et al. (1993a) were able to cultivate *Babesia caballi in vitro*. Advances have resulted in serum-free cultivation methods to be introduced. Zweygarth et al. (1999) utilized HL-1 to develop serum-free culture conditions for *B. caballi.* Martínez et al., (2016) used Advanced DMEM/F12 medium supplemented with a mixture of insulin, transferrin, and selenium for the adaptation and continuous culture of *B. bovis* in the absence of bovine serum. Thus, advances in a potential application of molecular biology and biochemistry will provide additional alternatives, making the future of adapting these parasites to *in vitro* growth broadly available.

Babesia genome structure and sequence

More information concerning the biology of *Babesia* parasites is needed to design novel and improved infection control methods. Important gaps of knowledge remain in our understanding of the biology of these parasites at all levels, but particularly at the molecular level and the mechanisms involved in parasite and host interactions. There is little information on the genomes of apicomplexans in general, and specifically for *Babesia* taxa of large and small ruminants (Gohil et al., 2013). To date, different *Babesia* spp. genomes have been sequenced and/or characterized for *B. bovis, Babesia orientalis*, *B. microti*, *Theileria parva,* and *T. equi.* Most of these studies are based on PCR and/or cloning while, some have utilized direct, deep sequencing of total genomic DNA (Gardner et al., 2005; Brayton et al., 2007; Huang et al., 2015; Silva et al.,2016).

The *B. bovis* Texas T2Bo isolate genome was released in 2007. This genome sequence was generated using a whole genome shotgun approach, confirming previous results from pulse field gel electrophoresis (Reddy and Dame, 1992; Jones et al., 1997; Brayton et al., 2007). Additionally, five *B. bovis* strains were sequenced by shotgun sequencing and assembled (Lau et al., 2011). These included the T2Bo Texas attenuated derivative, paired virulent and attenuated Argentina L17, and attenuated Australia T parasite lines (Lau et al., 2011).

The T2Bo genome confirms that the *B. bovis* parasite contains four chromosomes: i) Chromosome 1 which has two small assembly gaps and one physical gap, ii) Chromosomes 2 and 3 which were fully sequenced and are 1,729,419 and 2,593,321 bp in length, respectively, and iii) Chromosome 4 which contains an assembly gap that has not been unambiguously resolved. Thus, the 8.2 Mbp genome of *B. bovis* consists of four nuclear chromosomes and two

small extra-nuclear chromosomes for the apicoplast and mitochondria (Brayton et al., 2007). Approximately 60% of the *B. bovis* predicted genes do not yet have an assigned function.

The small genome size and analysis of enzyme pathways reveal a reduced metabolic potential and provide a better understanding of *B. bovis* metabolism. The previous genomic studies of *B. bovis* and *B. bigemina* were based on known regulatory mechanisms used by eukaryotic cells, combined with current high-throughput research technologies. The molecular tool of transcriptomics, proteomics, metabolomics, gene editing, and transfection systems were employed to understand the regulation of the gene expression (Laughery et al., 2009; Suarez and Noh, 2011; Bellgard et al., 2012; Wang et al., 2012). This regulation can be achieved on the transcriptional level using both genetic and epigenetic mechanisms. Moreover, it is likely that the activity of transcription factors essentially controls gene expression at the different parasite life cycle stages (Kensche et al., 2015). In addition, gene expression can also be regulated at the post-transcriptional and translational levels (Alzan et al., 2016).

Sequencing and annotation of the *B. bigemina* genome was initiated by the Trust Sanger Institute (Gohil, et al., 2013) with an estimated 10 Mbp, which is larger than the *B. bovis* genome (8.2 Mbp). A complete genome (13.84 Mbp) of *B. bigemina* strain Bond is now available (Piroplasm DB, http://piroplasmadb.org/piro/showApplication.do). Furthermore, the genome of the cattle tick vector, *Rhipicephalus (Boophilus) microplus*, is also in progress (Bellgard et al., 2012). It is expected to be 7.1 Gbp in size, which is more than twice the size of the human genome. Assembly of the tick genome is thus proving difficult, with more than 70% of the genome predicted to be repetitive DNA (Guerrero et al., 2010; Bellgard et al., 2012).

The zoonotic *Babesia divergens* (Rouen 1987; human isolate) was sequenced by a genome shotgun project and the sequences represent 10.7-Mb high-quality draft genome. The

data are deposited in the European Nucleotide Archive (Cuesta et al., 2014). Similarly, the *B. microti* nuclear genome was obtained with the whole-genome shotgun strategy approach (Cornillot et al., 2012). The genome is ~6.5 Mbp, which is 20% smaller than that of other piroplasms such as *B. bovis*

Recently, there have been efforts by the United States Department of Agriculture (USDA) to generate a National Agricultural Library with a section on Veterinary Pest Genomics to include sequences of all vectors and pathogens, a resource of data sets for the tick vector genome research community. This will provide integrated genomic and transcriptomic information through a single online tick genome sequencing resource with continuous updates (Guerrero et al., 2010; Andreotti et al., 2012; Guerrero et al., 2014; Pérez de León, 2017). Currently scientists can access such information at the Veterinary Pest Genomics Center (https://data.nal.usda.gov/ veterinary-pest-genomics-center) as well as through VectorBase, sponsored by the National Institute of Allergy and Infectious diseases Bioinformatics Resource Center (https://www.vectorbase.org).

Genomics tools in Babesia research application

Gene annotation, including their functional role and biological characterization, for most of the *B. bovis* genome that has been sequenced is still unknown. In general, the use of transcriptomic, proteomic and transfection approaches, alongside other techniques, is needed for the identification of genes that are differentially expressed in the various stages of the parasite life cycle.

Different molecular tools, such as the transfection method and gene expression in *Babesia*, may be used to characterize gene function, regulation and expression (Suarez and McElwain, 2010; Suarez et al., 2004). The availability of a stable transfection system for this

parasite will enable a wide range of applications in vaccine development studies. For instance, gene knock-out techniques can be used to determine virulence factors and to generate attenuated strains. In addition, strains could be labeled to track vaccinated animals compared to naturally infected animals. Recently, research focused on producing *Babesia* strains that express foreign tick antigens to be used as vaccines that can protect cattle against clinical babesiosis and also targeted tick antigens that interfere with *Babesia* transmission through the tick (Suarez and Noh, 2011). The employment of gene knockout technology and gene editing strategy for *T. gondii* and *P. falciparum* using the CRISPR/Cas9- based genome system indicates the feasibility of this genome editing system in various other organisms (Jimenez-Ruiz et al., 2014; de Koning-Ward et al., 2015). These developments in the regulation of gene expression at the genomic, transcriptional or protein level could be applied for *Babesia* spp. (Hakimi et al., 2016).

Anti-Babesia chemotherapeutic products and vaccines

Chemoprophylaxis is an important component in treatment and control of the bovine babesiosis but is, in general, short-lived and needs repeated administration, as well as being time-consuming and expensive. Successful *B. bovis* treatment usually relies on early diagnosis, followed by the administration of effective chemotherapeutic drugs. Currently, several antiprotozoal agents, as diminazene aceturate and imidocarb dipropionate, are effective in the treatment of bovine babesiosis, with the latter being the principal drug used as babesiacide for more the 20 years (Mosqueda et al., 2012). A high dose rate can provide short-term clinical protection of 120 days for *B. bovis*, about 8 weeks of protection for *B. bigemina*, and 3–6 weeks protection for *B. divergens* (de Waal and Combrink, 2006; Mosqueda et al., 2012).

The concern of drug residues in meat and dairy products following treatment was the cause of withdrawal of these products from most European countries (Zintl et al., 2003; de Waal

and Combrink, 2006; Mosqueda et al., 2012). Development of drug resistance by the parasite is a continuous major concern. The possibility of developing imidocarb-resistance, for example, due to prolonged exposure to a *Babesia* line was shown *in vitro* (Rodriguez and Trees, 1996).

Since the early use of the antiprotozoal agents in the control of protozoal infections, there was evidence of parasites developing drug resistance resulting in the call for replacement of disease treatment with an alternative prevention product using anti-*B. bovis* vaccines. Furthermore, acaricide resistance has been described in tick populations responsible for the transmission of Babesia to livestock, making it very challenging to control these pathogens in endemic areas of the world and contributing to significant economic losses (Avinash et al., 2017; Guerrero et al., 2014; Foil et al., 2004). Therefore, research directed toward the development of anti-babesia and anti-tick vaccines has been increasing in recent years (Almazan et al., 2018; Pérez de León et al., 2018; Petermann et al., 2017; Andreotti et al., 2012; Miller et al., 2012). The first use of a live attenuated *B. bovis* vaccine produced in Australia occurred in 1964 (Callow, 1964). It was discovered that multiple successive passages of virulent *B. bovis* strains through splenectomized calves would result in reduced virulence of the parasite (Callow and Mellors, 1966; Callow et al., 1979; Pipano, 1995). The use of such attenuated strains expanded for vaccine production have been successfully applied for control of bovine babesiosis and appear to be essential in the control programs in some countries such as in Australia, South Africa, Argentina and Brazil (de Castro, 1997). However, despite the efficacy of control programs based on the use of live attenuated vaccines in some countries, the vaccines also have a number of limitations including short shelf life and the requirement for cold storage, and the possibility of contamination with either bacteria or viruses (Timms et al., 1990; Wright et al., 1992). Other concerns include possible strain variation between natural infecting strains and the

vaccine strain, potentially rendering the vaccine ineffective (Bock et al., 1992). Finally, there is high cost of vaccine production due to the need of maintaining live animals free from specific diseases, whether the vaccine is produced from infected splenectomized calves and/or *in vitro* cultured parasites. If the latter, there are additional concerns associated with the technical difficulties of maintaining the cultivation system.

Later efforts aimed at identifying antigenic proteins that elicit humoral and cellular immunity, and investigation for new components as a subunit vaccine (Palmer and McElwain, 1995). Several attempts examined the *Babesia* Rhoptry Associated Protein-1 (RAP-1) gene family for its ability to induce immune protection as a subunit vaccine. All members of this family have a defined molecular feature making them highly immunogenic and they are expressed in the parasite blood stages as surface proteins (Suarez et al., 1991; Dalrymple et al., 1993; Ikadai et al., 1999). Despite the evidence of the induction of protective immunity, immunization with full or partial recombinant RAP-1 proteins produced a type 1 immune response that was insufficient to confer significant protection when experimentally immunized cattle were challenged with virulent *B. bovis* or *B. bigemina* (Rodriguez et al., 1996; Norimine et al., 2003).

Another approach for discovering proteins suitable as vaccine candidates is the family of Variable Merozoite Surface Antigens (VMSAs) which function in erythrocyte invasion by the parasite. A drawback is that the tandem organization of the *B. bovis* VMSA gene produces antigenic polymorphism among strains. Thus, recombinant MSA-1 as components of the VMSAs failed to produce immune protection when tested in immunization trials for bovine babesiosis on the basis of the ability of anti-MSA-1 antibodies to inhibit growth of the blood

stage in *in vitro* cultivation (Goff et al., 1988; Hines et al., 1992; Hines et al., 1995a, b; Suarez et al., 2000).

In another study, Australian researchers tested different combinations of designated recombinant *B. bovis* antigens, including the protein 12D3 (a short fragment of the rhoptry protein), the designated protein T21B4 (Bv60), and the antigen 11C5. Results showed that these antigens confer some protective immunity after *B. bovis* challenge (Wright et al., 1992). Furthermore, neutralization of sensitive epitopes was explored by immunization with combined recombinant MSA-2c, MSA-1 and 12D3. However, this was unable to prevent clinical disease upon challenge, although there was evidence indicative of immune protection against a mildly virulent strain of *B. bovis* (Alvarez et al., 2010). MSA-2b as a member of the VMSA family needs more investigation as a putative vaccine candidate since it is a protein on the surface of parasite merozoite exposed to host immunity defenses and has a putative role in host cell invasion (Genis et al., 2008; Dominguez et al., 2010).

Babesiosis epidemiology and economic impact

Bovine babesiosis is endemic in many tropical and subtropical areas including Australia, Africa, South and Central America, and Mexico, and the parasites are ranked as economically important tick-borne pathogens of livestock worldwide. It is prevalent wherever the tick vector is found. In Brazil, the annual economic losses due to reduced animal productivity caused by the cattle tick and its related diseases were estimated at about 3.24 U.S billion dollars (Grisi et al., 2014). In Mexico, total of ~ $574 U.S. million were lost as a consequence of effects on meat and milk production (Rodríguez-Vivas et al., 2017). Furthermore, there are losses due to abortion, mortality of cattle, and the costs of control programs and treatment. In Mexico, it is estimated that 75% of the national cattle herd is at risk of bovine babesiosis infection (Grisi et al., 2014;

Rodríguez-Vivas et al., 2017). In Puerto Rico, a national survey in 2000 reported ~ $6.7 US million economic losses due to bovine babesiosis, anaplasmosis, and the tick vector *R. microplus* (Cortés et al., 2005, Urdaz-Rodriguez et al., 2009).

Bovine babesiosis, also known as Texas tick fever, caused serious losses among the U.S. cattle population in the late 1800s to early 1900s. It was considered eliminated from the continental United States in 1943 after an extensive tick eradication program of the vectors *Rhipicephalus (Boophilus) annulatus* and *R. microplus* (Davey et al., 1980; Murrell and Barker, 2003). A permanent quarantine zone remains (PQZ) along the Texas border with Mexico; however, the U.S. remains under continuous threat of reemergence of the disease through the endemicity of babesiosis in neighboring Mexico and the continued presence of the tick vectors in the quarantine buffer zone. White-tailed deer (WTD) and its mobility across the landscape may cause chronic tick infestations in both cattle and WTD. The potential for such infestations on wildlife ungulate hosts, including introduced exotic antelope species such as nilgai (*Boselaphus tragocamelus*) raise additional concerns. Nilgai are native to India and were introduced to Texas in the early 1920s. Nilgai are known to contribute to the re-introduction and/or maintenance of populations of *Rhipicephilus* spp. in the U.S. (Cantu et al., 2007; Howell et al., 2007; Pérez de León et al., 2010; Pérez de León, 2017).

USA tick eradication program

Ticks reasonably are believed to be the oldest arthropods that have survived over 99 million years of evolution with minimal changes to their life processes (De La Fuente, 2003; Koonin et al., 2004; Mans et al., 2016; Peñalver et al., 2017). Controlling ticks is challenging because there are few natural enemies, it has a complex life cycle that includes free-living and on-host vertebrate stages, and because of its high reproductive capacity (De La Fuente, 2015).

The Cattle Fever Tick (CFT) is not native to the Americas but was likely introduced when Spanish colonialists brought infested livestock to Mexico in the 1500's (George, 1989).

Several attempts to limit or to decrease the size of tick populations have been remarkably unsuccessful, with exception of *Rhipicephalus (Boophilus)* spp. during the Cattle Fever Tick Eradication Program (CFTEP) achieved in the southern U.S. In 1906 (Pérez de León, et al., 2014) a mounted patrol of inspectors called "tick riders" first appeared as part of the cooperative State-Federal eradication campaign against the existing infestation of *Boophilus* ticks in the U.S. The Texas Animal Health Commission (TAHC) was established to mitigate problems affecting Texas livestock producers such as economic impacts of the Texas Cattle Fever Tick. Tick control by the application of acaricides was the centerpiece of this tick campaign because of its immediate effect by reducing tick populations. The Cattle Fever Tick Eradication Program had the advantage that *R. (Boophilus)* spp. complete their life cycle on a single preferred bovine host, thus, it was believed that employing pasture vacation would reduce the reproductive capability of the tick (Graham and Hourrigan, 1977). However, lately this practice has been proved non-effective due to the rise in infestations of ungulates other than cattle that are capable of supporting tick populations and is therefore no longer recommended by TAHC as a stand-alone option for control.

In 1943, the CFTEP was officially declared successful. A permanent quarantine buffer zone (PQZ) remains in Texas as an area ranging from 200 yards to 10 miles wide and approximately 500 miles long extending along the Rio Grande through eight South Texas counties to the Gulf of Mexico (Pérez de León et al., 2012). This area is maintained for quick detection and elimination of CFT entering Texas from Mexico in order to limit their spread outside the permanent quarantine zone. The United States Department of Agriculture Animal and

Plant Health Inspection Service Veterinary Services (USDA-APHIS-VS) inspects U.S. cattle in or near the PQZ, and its agents ride horses along the Rio Grande searching for wildlife or stray livestock that may cross the river into Texas from Mexico and be infested with CFT. They also apprehend any illegally entered (smuggled) Mexican livestock or native livestock that has crossed into Mexico and returned. Multiple agencies work together to find new effective and efficient ways to control ticks, such as dosing with Ivermectin-treated molasses as treatment methods for wildlife hosts (white-tailed deer) and using 4-post feeders to control tick burden on white-tailed deer (Skaggs et al., 2004) (www.tahc.texas.gov.; https://www.aphis.usda.gov).

The eradication of the vector and the elimination of bovine babesiosis in the U.S. is estimated to save the livestock industry 3 billion USD annually (Pérez de León et al., 2010). Therefore, it is important to modify the cattle Fever Tick control program as new tick challenges threaten and pose risks in the efforts to keep the national cattle free of babesiosis (Randolph, 2010; Pérez de León et al., 2012). Frequent CFT outbreaks in the U.S., outside of the quarantine zone in the last ten years increase the risk of re-establishment of bovine babesiosis in this country. Furthermore, the disease may spread beyond the southern state because of higher temperatures due to climate change in different portions of the U.S. providing a suitable environment for the vector tick (George, 2008). Development of pesticide resistance in vector tick populations in Mexico has been reported, likely resulting from misapplication of acaricides during prolonged periods of time (Avinash et al., 2017; Guerrero et al., 2013; Graf et al., 2004; Hopkins, 1994). In addition, potential wildlife ungulate hosts and introduced exotic antelope species (nilgai) may contribute to re-introducing and/or maintaining populations of *Rhipicephilus* spp. in the U.S. (Cantu et al., 2007; Howell et al., 2007; Baviskar et al., 2009; Pérez de León et

al., 2010). Thus, there is amplified risk of re-emergence of bovine babesiosis in the U.S. (Miller et al., 2013).

Despite the use of tick control measures, the availability of some live vaccines for preventing acute disease caused by *B. bovis*, and effective chemotherapeutics, bovine babesiosis remains poorly controlled in some parts of the world. Neither an effective vaccine against bovine babesiosis nor anti-babesial drugs are approved and/or available in the U.S., which limits therapeutic options for preventing disease or treating diseased animals should they occur. The estimated economic impact on the livestock industry could reach several billions of dollars if *Babesia*-infected vector ticks become re-established in Texas (Bram et al., 2002; Prez de León et al 2010 and 2012).

Successful tick control and eradication programs should be economically viable for the livestock industry, and appropriate legislation and adequate government, donor, and/or industry finance must be assured for uninterrupted progression and scientifically based tick control/eradication (Pérez de León et al., 2018; Pegram et al., 2000). In addition, there is a need for improved research on tick genetic resistance to acaricides in order to enhance the use of acaricides and their combinations. Therefore, there is a critical need to develop novel highly effective integrated pest management strategies that will control the populations of ticks and reduce the risk of bovine babesiosis in both endemic regions of the world as well as regions at risk of emergence or re-emergence. In addition, adequate livestock handling facilities and infrastructure is important to deal with current outbreaks. Furthermore, the acceptance of certain programs or strategies, including the logistical management of the quarantine areas, education of livestock owners, as well as providing sufficient extension services and information are all crucial to prevent re-infestation by CFT (Nolan, 1989; Myers et al., 1998).

Puerto Rico tick eradication program

Bovine babesiosis and the CFT are endemic in Puerto Rico (PR) causing significant morbidity and mortality, particularly among the dairy cattle breeds. (Pérez de León, 2017). The active eradication programs were instituted in 1936 and appeared successful by 1941, but then the tick emerged again in 1947 (Suthern and Comb, 1984; George, 1990). After five years of systematic application of acaricide dipping of cattle, and treating of equines, sheep and goats, Puerto Rico was declared free of *R. microplus* by 1952. In 1977 the tick was found again and by 1990 the entire island was under treatment. Over 50% of cattle on the island were considered free of *R. microplus* infestation by 1995, but infestations remain problematic. Thus, current control programs in Puerto Rico persist with the collaboration of the USDA-ARS.

There are many reasons for the failure of this eradication program. First, it is questionable whether or not the treatment methodology used was optimal because spraying instead of dipping was often used. Second, the program did not limit the movement of infested animals, thereby allowing re-introduction of the CFT to areas that had been clear. Finally, continued economic costs of the program were very high (about 11 million USD per year, at that time) without sound assurances that the program would succeed. Thus, these attempts to eradicate the CFT in Puerto Rico were seen as an example of government waste, and federal funding for the program was withdrawn (Bokma, 1996).

Development of tick vaccines

In the southern U.S., the cattle tick fever eradication program continues to rely on chemical pesticides for the control of *R. microplus.* This began with the early use of arsenical dip during the early 1900's (Graham and Hourrigan, 1977) which were replaced by the late 1960's with organophosphate and coumaphos in dipping vats for cattle and sprayers for horses,

respectively. Injectable Doramectin is currently approved for the use in cattle in the U.S., but requires a withdrawal period before cattle may be slaughtered for consumption. In Mexico, pyrethroids and amitraz whave been authorized for tick control since 1985 (Rosario-Cruz et al., 2009). However, the wide ranging use of amitraz, organophosphates and pyrethroids for treating tick infestations induced the appearance of multiple drug resistance, currently reported to involve organophosphates, pyrethroids, amitraz, and ivermectin in Mexico (Pérez-Cogollo et al., 2010; Fernández-Salas et al., 2012). In addition, resistance to organophosphates and pyrethroids has been reported in Texas (Miller et al., 2005). The difficulties in controlling ticks called for a critical need to replace current treatment with effective anti-tick vaccines.

The search for new tick antigens and new strategies for antigen selection is essential to improve the control of tick infestation through vaccination. The first approach to tick vaccination occurred when Trager (1939) reported the development of effective acquired immunity against *Dermacentor variabilis*. One of the great discoveries in this quest is the use of defined concealed antigens to produce an immunologic mechanism, which does not occur in the natural host parasite interaction (Willadsen and Kemp, 1988). This strategy was assessed by using a purified antigen, Bm86 glycoprotein, derived from the plasma membrane of tick gut epithelial cells, which results in destruction of the tick gut upon feeding on a vaccinated animal, killing the tick and providing partial protection against tick infestation (Willadsen et al., 1995). The vaccine, named TickGARD®, represents the first generation tick vaccine commercially available in Australia, and also has been used in other countries such as Brazil (Hungerford et al., 1995). CAVAC®, very similar product, was developed in Cuba and has been tested in Argentina, Cuba, Brazil, and Mexico (De La Fuente et al., 2007). Vaccination with Bm86 showed cross-protection against various other tick species, e.g., *Rhipicephalus (Boophilus) annulatus, Rhipicephalus*

(Boophilus) decoloratus, *Hyalomma anatolicum* and *Hyalomma dromedarii.* It has been demonstrated to elicit high anti-Bm86 specific antibodies, which are able to cause damaging effects on ticks as they feed on immunized cattle (De La Fuente et al., 2007; Merino et al., 2011). Although the vaccine has demonstrated some efficacy, there is a need to determine if the amino acid sequence of the commercial Bm86 vaccine is adequately effective in the face of geographic strain variation (Pérez de León et al., 2010).

The continuous development of better tick genomic databases, tools and other resources provided the foundation for a *R. microplus* genome-sequencing project (Canales et al., 2009; Guerrero et al., 2010). This research focused on mining the information available for different genomes to produce effective antigens that might be used for a cattle tick vaccine. Valuable resources include gene structure and expression, transcriptomics, and proteomics studies (Bostrom et al., 2008; Hill et al., 2009).

Guerrero et al. (2014) identified a common protein to be used as a vaccine candidate against *R. microplus* and *R. annulatus* which has been suggested to be the silver bullet in anti-tick vaccine development. A full-length aquaporin-like protein from *R. microplus* transcriptome datasets and another large part of one of those aquaporins were identified. Aquaporins, known also as water channels, regulate water transfer through the cell membranes. Membrane constriction pores contain the two-protein structure that acts as a size and charge selectivity filter for water, glycerol, and urea (Beitz et al., 2006). This water regulatory mechanism is required to be very efficient, since cattle ticks ingest large volumes of blood and then concentrate the blood constituents resulting in the rapid elimination of water (Megaw, 1974). One such aquaporin, RmAQP1, is a member of the *R. microplus* aquaporin family and was identified, expressed in the *Pichia pastoris* eukaryotic model, and purified as a recombinant protein (Guerrero et al. 2014).

This anti-cattle tick discovery was shown to be an efficacious vaccine antigen in Brazilian Holstein calves infested with larvae from *R. microplus* (Guerrero et al., 2014).

Tick saliva contains a wide range of bioactive tick proteins (multigene families) that modulate the host haemostasis, immune and inflammatory response (Chmelař et al., 2016). Tick vaccines based on tick salivary antigens may induce an immune response, and then exposure of vaccinated animals to saliva during natural tick challenges would boost immunological memory, thus avoiding the problems of declining immunity seen with vaccines formulated with non-salivary antigens (Chmelař et al., 2016; De La Fuente et al., 2015).

Global and climate change

Accumulation of environmental and climatic changes together with habitat modifications ascribed to human activity are all referred to as globalization (Sutherst, 2001; Camill, 2010). Thus, global change poses fundamental threats to human and animal health. In particular, emerging and reemerging diseases transferred by ticks have been expanding in different areas of the world due to changes in the spatial distribution and abundance of tick species, and their associated pathogens (Sutherst, 2001; Léger et al., 2013).

Among parasites, ticks are considered highly sensitive and likely to be affected by climate change and climate variability (Kingsolver, 1989). Ticks are very dependent on their environment in both its biotic and abiotic activity for their survival and reproduction (Randolph, 2010). Ticks are very vulnerable to environmental conditions (e.g. humidity, temperature, and precipitation) during the free-living egg and/or larvae parts of their life cycle (Estrada-Peña et al. 2004; Gray 1991; Vassallo et al., 2000; Esteve-Gassent et al., 2015, 2016). The local existence of ticks depends on the presence and abundance of their vertebrate hosts since they require blood meals from their hosts (Gilot and Pérez-Eid, 1998; Gray 1998). Changes in host populations,

including the reservoir host species, may shift the ecology and evolution of the tick biology, which consequently alters the epidemiology of pathogens transmitted by these ticks. This may mean continued maintenance of the pathogens for transmission to vectors and can result in an increase in both tick density and pathogen prevalence (Randolph, 2010; Olson and Patz, 2011).

Many other drivers of global change may affect tick management such as new technology, landscape modifications, and livestock transportation (Reisen, 2010; Esteve-Gassent et al., 2015, 2016). Ticks can easily be spread though transport of the infested host, and then expand geographically according to the availability of the host and appropriate environmental conditions. Spreading by transportation would be successful when it is less time in duration than the tick life cycle stage in the case of a multi-host tick species (Barré and Uilenberg 2010). Therefore, the effectiveness of spread by host transportation is facilitated in one-host ticks. Thus, the tick *R. annulatus*, originally a parasite of Asian bovid species from India and Indonesia, has spread into tropical and subtropical regions during the past 150 years, due to the introduction of European cattle (*Bos taurus*) to tropical areas. Cattle in these regions were almost incapable of mounting efficient immune responses to *R. microplus* infestations. As a result, the introduction of *R. annulatus* became one of heaviest economic impacts in many agricultural ecosystems (Frisch, 1999; Labruna et al., 2009).

The ability of ticks to adapt to a new host, resulting in the evolution of tick biodiversity, could result in exploitation of a novel host. For example, in 1942 *R. microplus* invaded New Caledonia after an accidental introduction of a few infested cattle from Australia. The cattle tick quickly adapted to the rusa deer (*Cervus timorensis rusa*) on the island, even though this deer was not a preferred host for this tick (Barre´ et al., 2001). After about 200 generations, the bovid

tick population was able to adapt to exploit this host and now can be seen in sympatric ranges with the cattle tick population but exhibits genetic differences (de Meeuˆs et al., 2010).

Global change is influencing the Cattle Fever Tick eradication program in south Texas, and involves shifting in the regional ecology, including the availability of the suitable hosts, environmental changes, human impact, acaricide resistance, and the infestation of different wild life animals as alternative hosts (Pérez de León et al., 2012).

Any environmental change that removes a barrier previously effective may negatively impact control measures in place (Estrada et al., 2005). For example, during droughts, when rainfall declines to amounts that can't maintain the Rio Grande at levels that serve as a good physical barrier to prevent or slow livestock and wildlife movement into the U.S., ticks may breach that normal barrier (Pérez de León et al., 2012, Giles et al., 2014) and reach the PQZ.

In the early 1940s, although there was no scientific evidence showing a role for white-tailed deer in sustaining and/or spreading CFT, 20,000 of these animals were slaughtered in Florida in an attempt to control their populations. Multiple events in Texas by 1968-1970's drew attention to the potential importance of white-tailed deer in *R. annulatus* ecology. Later, it was determined that deer are a physiologically suitable host and capable to maintain the CFT population in the absence of cattle (Graham et al., 1972; George, 1990). It has been demonstrated that movement of ticks with white-tailed deer from Mexico across the river is a source of CFT infestations in Texas (Pérez de León et al., 2012; Busch, et al., 2014).

CHAPTER II

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF A HAEMOGREGARINE IN THE ALLIGATOR SNAPPING TURTLE, *MACROCHELYS TEMMINCKII* (TESTUDINES: CHELYDRIDAE)[1]

Introduction

The alligator snapping turtle, *Macrochelys temminckii* Troost in Harlan, 1835 (Testudines: Chelydridae), is characterized by sharp claws, spiked carapacial ridges, and huge head with strongly hooked beak. It is distributed in North America across the southeastern USA and is endemic to river systems that drain into the Gulf of Mexico from the Suwannee River in Georgia and Florida, and from the Brazos River in Texas (Powell et al., 2016). It is the largest species of freshwater turtle in North America, weighing up to 135 kg. It is highly aquatic although oviposition occurs on land. The alligator snapping turtle is non-migratory and a top trophic-level predator (Boundy and Kennedy, 2006; Ernst and Lovich, 2009; Ewert, 1976).

Haemogregarines are widely distributed blood parasites among aquatic turtles and have been reported in North America, Europe, Asia, and Australia (Telford, 2009). Leeches are thought to be the invertebrate hosts and the transmission vectors for aquatic vertebrate hosts, while ticks are the transmission vectors for parasites of terrestrial reptiles (Telford, 2009; Cook et al., 2009; Merino et al., 2009; Siddall and Desser, 1991). In the USA, *Haemogregarina* spp. have been investigated in freshwater turtle populations in Louisiana, Georgia, Tennessee, Kentucky, and Texas (Acholonu, 1974; Davis and Sterrett, 2011; Edney, 1949; Strohlein and

[1] Reprinted with permission from "Molecular and morphological characterization of a haemogregarine in the alligator snapping turtle, *Macrochelys temminckii* (Testudines: Chelydridae)" by Amer Rasool Alhaboubi, Dana Pollard and Patricia Holman, 2017. *Parasitology Research*, 116, 207-215, Copyright 2016 Springer-Verlag Berlin Heidelberg

Christensen, 1984; Wang and Hopkins, 1965) and particularly in the alligator snapping turtle in Arkansas, Georgia, and Florida (McAllister et al., 1995; Telford et al., 2009). Haemogregarines are intraerythrocytic apicomplexans that belong to the suborder Adeleorina of the family Haemogregarinidae. *Haemogregarina* species have indirect life cycles. They infect a wide variety of vertebrate hosts such as mammals, birds, fishes, snakes, crocodilians, lizards, and turtles in which they undergo asexual cycles of merogony, gametogony, syngamy, and sporogony (Barta et al., 2012; Siddall and Desser, 1992). The various types of meronts and merozoites that arise may either initiate further rounds of merogonic replication or differentiate into gamonts (Barta et al., 2012; Smith, 1996). The invertebrate transmission vectors in which the sexual stages take place include ticks, mites, other arthropods, and leeches (Telford Jr, 2009). Although the complete life cycles for *Haemogregarina balli* in Nearctic snapping turtles and *Haemogregarina stepanowi* Danilewsky, 1885, in the European pond turtle have been described, data regarding the complete life cycles for many of these organisms are lacking (Siddall and Desser, 1991, 1992; Telford, 2009). However, intraerythrocytic stages for a number of *Haemogregarina* spp. have been described in various hosts from different parts of the world (Barta et al., 2012; Davis and Sterrett, 2011). Nearly 300 haemogregarine species are named in previous studies based on morphological and/or biological features, such as the size and the shape of the stages in the host erythrocytes (Levine, 1982; Perkins and Keller, 2001). However, morphological similarities and the lack of detected life cycle stages, especially the identification of the vector and morphology of the parasite in the vector, have prevented an accurate identification of the different infective forms (Levine, 1982). Numerous *Haemogregarina* spp. have been reported in turtles and tortoises globally, but descriptions of chelonian hematozoa in the USA are limited (Acholonu, 1974; McAllister et al., 1995; McAllister, 2015; Strohlein and

Christensen, 1984; Telford et al., 2009; Wang and Hopkins, 1965) with some including photomicrographs and selected measurements. New molecular tools combined with morphological approaches provide acceptable differential diagnoses and taxonomy of *Haemogregarina* spp. (Lv et al. 2015). To date, the 18S ribosomal RNA (rRNA) gene has been utilized in characterization, identification, and prevalence as well as in taxonomic relationship studies among apicomplexan parasites in reptiles, including freshwater turtles (Dvořáková et al., 2015; Kopečná et al., 2006; Kvičerová et al., 2014; Maia et al., 2014; Rakhshandehroo et al., 2016; Telford et al., 2009). This study entails the first known *Haemogregarina* sp. identified in the alligator snapping turtle (*M. temminckii*) in Texas and includes both morphological and molecular descriptions of this parasite. Moreover, this report provides the first DNA sequence data for a *Haemogregarina* sp. in the USA.

Materials and Methods

An alligator snapping turtle was found in early January 2015 laying by the side of a road near Tyler, Texas, and was taken to the Caldwell Zoo in Tyler. The turtle was drastically underweight. No leeches were present although examination of a stained blood film revealed the presence of haemogregarine-like inclusions in the red blood cells. Unfixed blood films were sent to the Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, for parasite identification. A blood film was methanol fixed and Giemsa stained (AccuStain, Sigma) for microscopic examination by light microscopy at ×1000 magnification under immersion oil. The parasitemia was estimated based on examination of 1000 erythrocytes. Scanning and morphometric data for the blood stage parasites were obtained, and images were captured using an Olympus IX70 microscope (Olympus America, Inc. Center Valley, PA) equipped with Spot Software 5.2 Macro-

Photography© 2016 SPOT Imaging (Diagnostic Instruments, Inc. Avon, MA). The length and width for multiple examples of each developmental stage observed were measured in micrometers and the mean and standard deviation determined. The range in length and width for each stage was recorded. The observed morphology of the blood stage parasites in this study was compared with published morphological data on turtle haemogregarines (McAllister 2015; Telford, 2009; Telford et al., 2009).

DNA isolation and amplification

The blood film from one slide was scraped into 100 µl of Dulbecco's phosphate-buffered saline (PBS) using a sterile scalpel blade. DNA was extracted from the blood following the manufacturer's protocol (FlexiGene DNA Extraction kit, Qiagen, Redwood City, CA, USA). The extracted DNA concentration was determined (NanoDropND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and adjusted to approximately 100 ng/µl.

Forward primer HemoFN (5′-CCGTGGTAATTCTAGAGCTAATACATGAGC-3 ′) and reverse primer HemoRN (5′-GATAAGGTTTACGAAACTTTCTATATTTA- 3 ′) were designed from an alignment of haemogregarine *18S rRNA* gene sequences in the GenBank® database to amplify a gene fragment of ~1550 bp. The amplification reaction was performed according to the manufacturer's instructions (Phusion® High-Fidelity DNA polymerase, FINNZYMES, New England Biolabs, Inc., Ipswich, MA, USA) in a final volume of 25 µl containing ~100 ng of DNA. A negative control reaction (no DNA) was included. The amplification profile was initial denaturation at 98 °C for 2 min followed by 35 cycles of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min followed by hold at 4 °C (Labnet MultiGene Thermal Cycler, Woodbridge, NJ, USA). All amplicons were electrophoresed through a 1% agarose gel alongside a 200 bp marker

(BioDL200 BioFlux, Bulldog Bio, Portsmouth, NH, USA), stained with ethidium bromide, and visualized under ultraviolet transillumination.

Cloning and phylogenetic analyses

Appropriately sized amplicons of ~1550 bp were directly ligated within 24 h of PCR into the pJET1.2/blunt plasmid vector (CloneJET PCR Cloning Kit, Fermentas, Inc., Burlington, Ontario, Canada) and chemically competent *Escherichia coli* cells (TOP 10 One Shot®INVαF; Invitrogen, Grand Island, NY, USA) transformed following manufacturers' instructions. Twenty transformed colonies were randomly chosen for colony PCR to verify the insert, and five verified colonies were expanded in overnight cultures. Plasmid DNA was extracted from the cultures (EZ-10 Spin Column Plasmid DNA Minipreps Kit, Bio Basic Inc., Amherst, NY, USA), quantified as above and adjusted to a concentration of approximately 150 ng/µl for automated bidirectional sequencing (Eton Bioscience Inc, San Diego, CA, USA) using pJET1.2 sequencing primers (CloneJET PCR Cloning Kit). The resulting sequences were analyzed using Sequencher Version 5.1 and manually trimmed to remove ambiguous or unreadable data. Chromatogram-based contiguous sequences were generated for each of the five clones, and similarities to *18S rRNA* gene sequences in the GenBank® database were determined using the NCBI Basic Local Alignment Tool (BLAST) (Altschul et al. 1990). The obtained sequences were deposited in the NCBI GenBank® database. The sequences were aligned using Muscle (http://www.ebi.ac.uk/Tools/msa/muscle) with additional sequences of *Haemogregarina* species and those of closely related genera, and *Cryptosporidium serpentis* as the outgroup, selected from the NCBI GenBank® database (Table 2.1). The sequences in the Muscle alignment were trimmed to equivalent lengths BioEdit (Hall 1999), and the final alignment was used to construct a maximum likelihood tree in DIVEIN (https://indra.mullins.microbiol.washington.

edu/DIVEIN/diver.html) under the default settings. The resulting tree was viewed and appropriately labelled using Mega 6.0 (Tamura et al. 2013).

Table 2.1 List of taxa used to generate phylogenetic tree. GenBank® accession numbers and relevant references are included (Reprinted with permission from Alhaboubi et al., 2017a).

Taxon	Accession No.	Reference
Adelina dimidiate	DQ096835	Kopečná et al. 2006
Cryptosporidium serpentis	AF093499	Xiao et al., 1999
Dactylosoma ranarum	HQ224957	Barta et al., 2012
Dactylosoma ranarum	HQ224958	Barta et al., 2012
Haemogregarina balli	HQ224959	Barta et al., 2012
Haemogregarina pellegrini	KM887508	Dvořáková et al., 2015
Haemogregarina pellegrini	KM887509	Dvořáková et al., 2015
Haemogregarina sacaliae	KM887507	Dvořáková et al., 2015
Haemogregarina stepanowi	KF257929	Dvořáková et al., 2014
Haemogregarina stepanowi	KT749877	Dvořáková et al., 2014
Haemogregarina stepanowi	KF257927	Dvořáková et al., 2014
Haemogregarina sp.	KF257923	Dvořáková et al., 2014
Haemogregarina sp. (Gabon)	KF257924	Dvořáková et al., 2014
Haemogregarina sp.	KF257925	Dvořáková et al., 2014
Haemogregarina sp. Clone 2	KX507246	Alhaboubi et al., 2017a
Haemogregarina sp. Clone 4	KX507247	Alhaboubi et al., 2017a
Haemogregarina sp. Clone 6	KX507248	Alhaboubi et al., 2017a
Haemogregarina sp. Clone 7	KX507249	Alhaboubi et al., 2017a
Haemogregarina sp. Clone 8	KX507250	Alhaboubi et al., 2017a
Hemolivia mariae	KF992711	Kvičerová et al., 2014
Hemolivia mariae	KF992712	Kvičerová et al., 2014
Hemolivia mauritanica	KF992705	Kvičerová et al., 2014
Hemolivia mauritanica	KF992706	Kvičerová et al., 2014
Hemolivia mauritanica	KF992710	Kvičerová et al., 2014
Hemolivia stellata	KP881349	Barta et al., 2012
Hemolivia sp.	KF992714	Kvičerová et al., 2014
Hepatozoon ayorgbor	EF157822	(Sloboda et al., 2007)
Hepatozoon cf. *clamataecf.*	HQ224963	Barta et al., 2012
Hepatozoon domerguei	KM234648	(Barta et al., 2012)
Hepatozoon felis	AY620232	(Criado-Fornelio et al., 2006)
Hepatozoon sp.	HQ224960	Barta et al., 2012
Hepatozoon sp.	FJ719813	(Merino et al., 2009)

Results

Host

The alligator snapping turtle was not treated. It is currently in good health (Fig. 2.1) and remains in residence at the Caldwell Zoo, Tyler, Texas.

Morphology

Intraerythrocytic *Haemogregarina* sp. forms with morphology typical of different blood stages were found on microscopic examination of a Giemsastained blood film (Fig. 2.2). The parasitemia was <1%. No extracellular organisms were identified, nor were organisms found within white blood cells. Among the intraerythrocytic stages observed were trophozoites, premeronts, meronts, and gamonts (Figs. 2.2 and 2.3). Trophozoites were the smallest forms and occurred individually in the erythrocyte with length × width dimensions of 8.75 ± 0.5 × 3.75 ± 1.4 μm (range, 8–9 × 3–5; $n = 4$) (Fig. 2.2a–c). Trophozoites possessed a prominent eccentric nucleus and vacuolated cytoplasm. Premeronts were the most common form seen in the blood film (Fig. 2.2d–f). The parasites were elongated with a central or slightly eccentric nucleus located toward the side of the parasite. Premeronts measured 13.42 ± 0.80 × 5.95 ± 0.41 μm (range, 12–15 × 5–7; $n = 25$). Meronts were elongated and slightly curved, measuring 11.1 ± 1.7 × 4.9 ± 0.7 μm (range, 10–14 × 4–6, $n = 10$), and contained a single nucleus (Fig. 2.3a–b) or multiple irregular nuclei (Fig. 2.3c–f). The immature gamont possessed a nucleus located toward or at one end (Fig. 2.3g–i) and were 11.6 ± 0.8 μm × 5.1 ± 0.4 μm (range 11–13 × 5–6, $n = 6$).

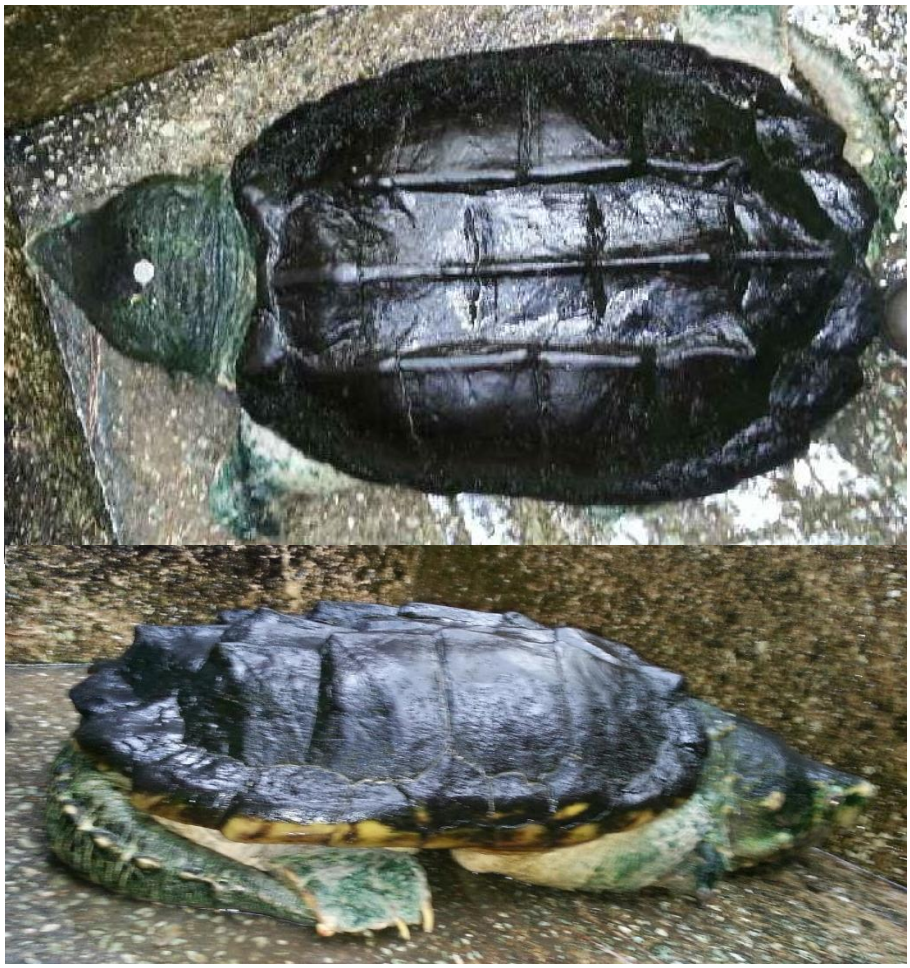

Fig. 2.1 Dorsal (top panel) and lateral (bottom panel) views of the alligator snapping turtle *Macrochelys temminckii* infected with *Haemogregarina.* A quarter placed on the turtle's head demonstrates his size (top panel) **(**Reprinted with permission from Alhaboubi et al., 2017a).

Gamonts were slender and recurved with twin folded approximately equal limbs. The gamont total length was 33 ± 7.02 × 3.3 ± 1.4 μm (range, 26-40 × 3-4, *n* = 4) with LW 133.75. ± 39.4 μm2 (78–160) and L/W 9.6 ± 0.9 (8.6–10.8). The length of the gamont folded limbs ranged from 14 to 15 μm with a width across both limbs of 6-8 μm (Fig. 2.3j, k). The nucleus was located at one end of the structure in the bend of the limbs. Encapsulated gamonts were rare and appeared as non-staining bodies (Fig. 2.3l).

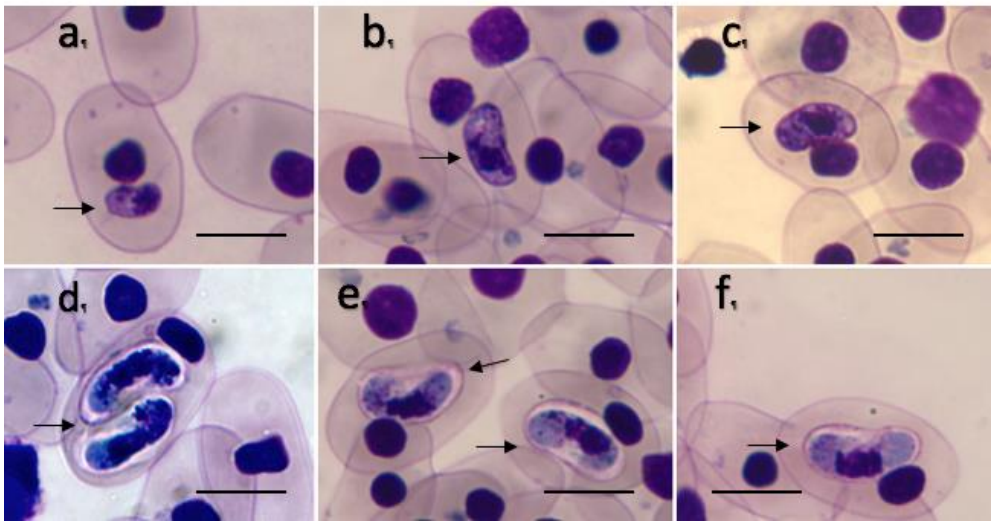

Fig. 2.2 Morphology of *Haemogregarina* sp. trophozoite (a–c) and premeront (d–f). Intraerythrocytic parasites are indicated by black arrows. ×1000, Giemsa stain. Scale bar =10μm **(**Reprinted with permission from Alhaboubi et al., 2017a).

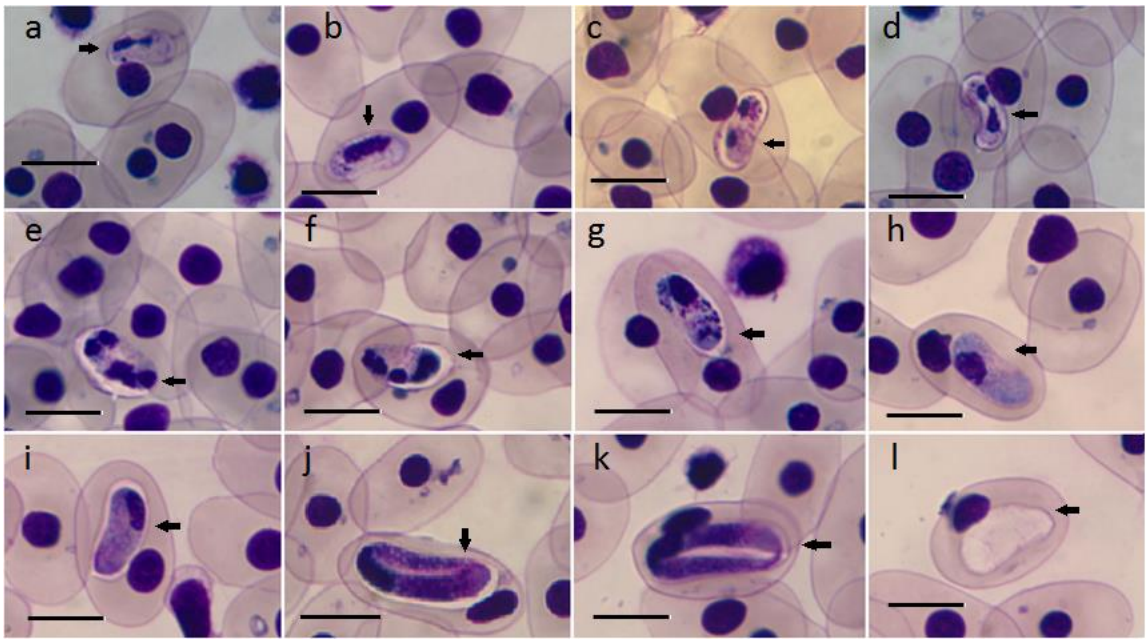

Fig. 2.3 Morphology of *Haemogregarina* sp. meronts and gamonts. Meront with a single nucleus (a, b), with 2 nuclei (c, d), and multi-nucleated (e, f) are shown. Immature gamont (g-i), gamont (j, k), and encapsulated gamont (l) are shown. Intraerythrocytic parasites are indicated by black arrows. ×1000, Giemsa stain Scale bar = 10μm **(**Reprinted with permission from Alhaboubi et al., 2017a).

A voucher Giemsa-stained specimen slide has been deposited in the Systematics Research Collections, University of Nebraska State Museum, Lincoln, NE, under Accession Number P-2016-057.

Molecular characterization

Genomic DNA from the alligator snapping turtle blood film was obtained with a concentration of 696 ng/µl. The primary PCR successfully amplified the *18S rRNA* gene region with the designed primers, yielding amplicons of the expected size of ~1550 bp. Twenty transformed colonies were verified to contain the proper insert by PCR and five (clones 2, 4, 6, 7, and 8) were selected for sequence analysis. The cloned sequences were 1555 bp in length and varied in sequence from one another, ranging from 99.3% to 99.9% identity (Table 2.2). Clone 6 was the most variable, differing from the other clones in 8 to 10 nucleotide positions (99.3% - 99.5% identity).

Table 2.2 Matrix of differences in *Haemogregarina* 18s rDNA nucleotide sequences. Upper matrix (unshaded) shows percent differences in nucleotide sequences, and the lower matrix (shaded) shows the number of base differences between clones of *Haemogregarina* sp. from the alligator snapping turtle **(**Reprinted with permission from Alhaboubi et al., 2017a).

Clone	**2**	**4**	**6**	**7**	**8**
	KX507246	**KX507247**	**KX507248**	**KX507249**	**KX507250**
2	100	99.8	99.4	99.6	99.9
3	1	100	99.3	99.7	99.9
6	9	10	100	99.3	99.5
7	4	5	10	100	99.7
8	1	1	8	3	100

The 18S rDNA 1555-bp insert sequence of the five clones all showed ~96% identity to *H. balli* Paterson and Desser, 1976, *Hepatozoon* sp., and *Hemolivia stellata* Petit et al., 1990 (GenBank® accession nos. HQ224959, FJ719813, and KP881349, respectively) by BLAST analysis. Higher identity was found between *Haemogregarina* sp. (KR006985) from Iran (99% identity with clones 2, 6, and 8; 98% identity with clones 4 and 7) by BLAST analysis of a 774 bp segment corresponding to that deposited for KR006985. The obtained sequences were deposited in the NCBI Genbank® database under accession numbers KX507246, KX507247, KX50724 8, KX50724 9, and K X507250 for *Haemogregarina* sp. clones 2, 4, 6, 7, and 8, respectively.

The five cloned sequences were aligned with 26 *18S rRNA* gene sequences from closely related taxa, and from *Adelina dimidiata* and *C. serpentis* (Table 2.1). The final aligned length was 1382 bp after trimming sequences as needed to uniform corresponding fragments. The maximum likelihood phylogenetic tree generated from the final alignment shows three well supported clades of the adeleorinid parasites infecting a variety of hosts (amphibians, reptiles, and mammals) (Fig. 2.4). The first clade contains two isolates of *Dactylosoma ranarum* (Kruse, 1890), the second contains *Haemogregarina* spp., and the third clade includes both *Hemolivia* spp. and *Hepatozoon* spp. but branches into two separate clusters, one holding the seven *Hemolivia* spp. isolates and one holding the five *Hepatozoon* spp. isolates. The tree topology shows the *Haemogregarina* sp. cloned sequences from this study within the *Haemogregarina* spp. clade, but in a separate branch clustered together, distinct from the previously reported sequences (Fig. 2.4).

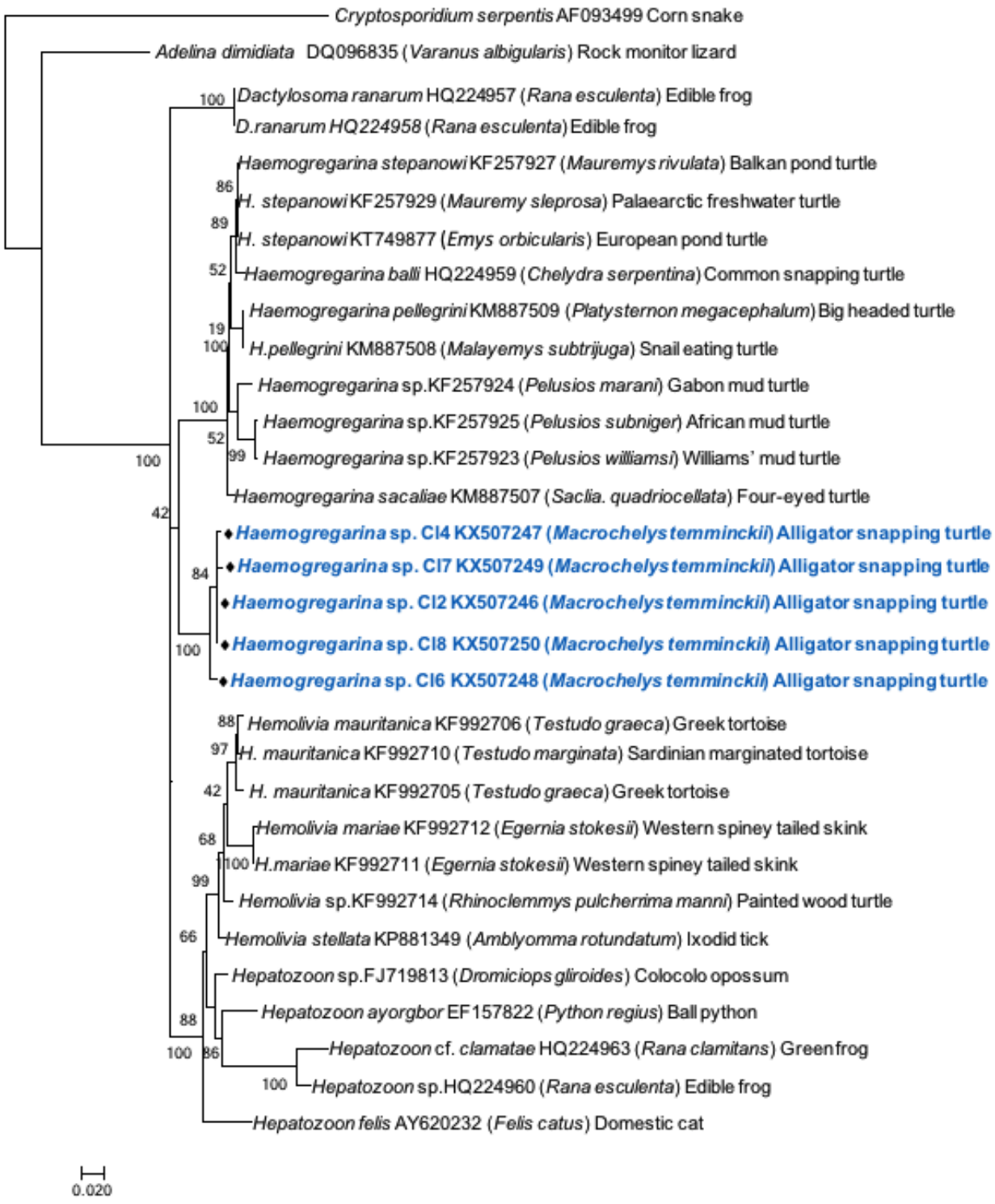

Fig. 2.4 Maximum likelihood phylogenetic tree based on *18S rRNA* gene sequences. Bootstrap support is shown at branches. Cloned sequences for the *Haemogregarina* sp. found in the alligator snapping turtle in this study are indicated by black diamond **(**Reprinted with permission from Alhaboubi et al., 2017a).

Discussion

The alligator snapping turtle in this report was found in the winter in an emaciated state. The finding of intraerythrocytic parasites caused speculation regarding the possible impact of the infection on the turtle's health. However, generally infections by haemogregarine blood parasites are considered benign, despite being persistent (Davis and Sterrett 2011). Davis and Sterrett (2011) suggest that the high incidence of haemogregarine infections in aquatic turtles, averaging 70% in North American published reports, is evidence of the benign effect of these parasites on these hosts.

Intracellular haematozoa (Order Eucoccidioida, suborder Adeleorina) were found at a low parasitemia in erythrocytes from the alligator snapping turtle in this study. The morphology and morphometric data for the parasite forms indicate conformity to *Hemogregarina* spp. previously reported in freshwater turtles in the USA (Davis and Sterrett 2011; Telford et al. 2009). The intraerythrocytic premeront and immature gamont in the current report are similar to the haemogregarine small and large forms, respectively, described in *M. temminckii* in Arkansas (McAllister et al. 1995). However, the medium form described as resembling a microgamont by McAllister and others (1995), was not seen in the blood film from the Texas alligator snapping turtle in this study. The various trophozoites, premeronts, meronts, and gamonts observed in the current study generally resemble those seen in *Haemogregarina macrochelysi* n. sp. (Apicomplexa: Haemogregarinidae) from alligator snapping turtles in the states of Georgia and Florida (Telford et al. 2009). However, Telford et al. (2009) described *H. macrochelysi* n. sp. as set apart from all other described species by macromeronts that contained 150 or more nuclei in circulating blood. These were not seen in the *Haemogregarina* sp. in this current study; no more than four nuclei within a meront was observed. Another distinguishing feature between the two

haemogregarines from this host is the appearance of the meront nuclei. Multiple nuclei within a meront were uniform in shape and size in *H. macrochelysi* n. sp., whereas they were irregular in the *Haemogregarina* sp. In the current study, encapsulated gamonts were observed, which were not reported for *H. macrochelysi* n. sp. (Telford et al. 2009). We were unable to determine the invertebrate vector for the *Haemogregarina* turtle in this study. Whether the haemogregarine in the alligator snapping turtle in this current study is conspecific with *H. macrochelysi* n. sp. or with the haemogregarine previously described in the alligator snapping turtle in Arkansas remains to be definitively determined. While molecular comparisons would undoubtedly resolve this question, no genetic data are available for either the Arkansas isolate or *H. macrochelysi* n. sp. at this time.

Molecular characterization of the *Haemogregarina* sp. in the current study was based on *18S rRNA* gene sequence analysis of five clones. As previously reported in cloned haemogregarine *18S rRNA* genes (Perkins and Keller 2011), sequence variation was found among the five *Haemogregarina* sp. clones and none was identical to each other. Nonetheless, the clones were more like one another than to other reported 18S rDNA sequences for haemogregarines. Interestingly, the highest sequence identity (98-99%) was to an unnamed *Haemogregarina* sp. found in the Caspian freshwater turtle *Mauremys caspica* in Fars Province in southern Iran (Rakhshandehroo et al. 2016). Only a short *18S rRNA* gene sequence (774 bp) is available for this parasite (GenBank® accession no. KR006985), precluding its incorporation in the phylogenetic analysis. *H. balli* found in the common snapping turtle *Chelydra serpentina serpentina* Linnaeus, 1758, might be expected to be more closely related to a *Haemogregarina* sp. of an alligator snapping turtle, but a comparison of this corresponding *18S rRNA* gene fragment shows only 96.3% identity between the two. Unfortunately, there are no genetic data

from US haemogregarines available for comparison to those obtained in this study for the *Haemogregarina* sp. in the alligator snapping turtle.

This study entails morphological and molecular characterization of a *Haemogregarina* sp. in an alligator snapping turtle in Texas. The findings of this study compare with available morphological data of haemogregarine developmental stages previously described and provide the first genetic data for a *Haemogregarina* sp. in this hemisphere.

CHAPTER III

RECOVERY OF BOVINE *BABESIA* SPP. AFTER LONG-TERM CRYO STORAGE AND COMPARISON OF BOVINE DONOR ERYTHROCYTES AND SERUM [2]

Introduction

Babesia bovis and *Babesia bigemina* are highly pathogenic blood borne tick transmitted protozoans that are the causative agents of bovine babesiosis in the Americas (Levine, 1971). Bovine babesiosis is characterized by clinical signs including fever, malaise and inappetence, severe anemia, icterus, and enlarged spleen, lymph nodes, and liver (Levine, 1985). The disease caused by *B. bovis* is often considered more detrimental than that caused by *B. bigemina* because the infected erythrocytes can pass the blood-brain barrier causing cerebral babesiosis with ensuing neurological signs. Bovine babesiosis may result in abortions, loss of milk or meat production, loss of draft power, or even death (Bock et al., 2004). The disease is endemic in many tropical and subtropical areas including Australia, Africa, South and Central America, and Mexico, and the agents are ranked as economically important tick-borne pathogens of livestock worldwide. Bovine babesiosis affects adult animals more severely than young, which can lead to enzootic stability in regions where the climate is favorable to the vector tick and cattle and *Babesia* sp. are also present. The tick transmits Babesia to calves, which become infected but do not show clinical signs and remain subclinical carriers (Levine, 1985). Bovine babesiosis was considered eliminated from the United States in 1943 after an extensive tick eradication program

[2] Reprinted with permission from "Recovery of bovine *Babesia* spp. after long-term cryostorage and comparison of bovine donor erythrocytes and serum" by Alhaboubi, McCormack, Gustafson and Holman. 2017, Veterinary Parasitology, 116, 207-215, Copyright 2017 Elsevier B.V.

targeting the vector ticks *Rhipicephalus (Boophilus) annulatus* and *Rhipicephalus (Boophilus*) *microplus* (Hejl, 1976; Murrell and Barker, 2003).

A permanent quarantine zone remains along the Texas border with Mexico, however the U.S. remains under continuous threat of reemergence of the disease through the endemicity of babesiosis in neighboring Mexico and the continued presence of the tick vector in the buffer zone (Howell et al., 2007; Pérez de León et al., 2010; Busch et al., 2014). The availability of continuous cultures of *B. bovis* and *B. bigemina* provides an unlimited laboratory source of parasites free of adventitious organisms for numerous purposes such as molecular and biochemical studies, studies of the parasite biology, anti-babesial drug testing, and diagnostic test and vaccine development (Yamagishi et al., 2014; Wang et al., 2014; Mossaad et al., 2015). *Babesia bovis* attenuates as it is subcultured *in vitro* thus cultured parasites have been employed as attenuated vaccine (Ojeda et al., 2010). Importantly, use of cultures reduces the use of live animals in research such as in preliminary testing of therapeutic drugs for anti-babesial activity (Aboge et al., 2015; Munkhjargal et al., 2016). *Babesia bovis* and *B. bigemina* are closely related taxonomically and share the same life cycle, host range, and tick vectors (Levine, 1985; Ellis et al., 1992; Chauvin et al., 2009). Thus, the introduction of continuous cultivation of the erythrocytic stage of *B. bovis* provided an avenue to culturing *B. bigemina* as well (Levy and Ristic, 1980; Vega et al., 1985a). Levy and Ristic (1980) introduced the microaerophilous stationary phase (MASP) culture system for *B. bovis*, premised on previous culture work with plasmodium (Trager and Jensen, 1976). The success of the cultures is dependent upon maintaining a low oxygen tension in the settled erythrocyte layer in addition to providing adequate nutrients and erythrocytes for parasite invasion and multiplication.

Erythrocytic stage *B. bovis* or *B. bigemina* are established *in vitro* using blood from an infected bovine for initiation of cultures. Erythrocytes and serum from a normal uninfected adult bovine donor are used to maintain the cultures and adapt the parasites to continuous propagation (Levy and Ristic, 1980; Vega et al., 1985a, 1985b). Due to the differences between individual animals, it is necessary to select a suitable donor animal for routine blood supply (Canning and Winger, 1987). The washed donor erythrocytes collected in anticoagulant or by defibrination may be stored at 4 °C for 2 or 4 weeks, respectively, for use and the serum may be stored at -20 C or - 80 °C for several months or longer (Canning and Winger, 1987). Low temperature cryopreservation allows the long-term storage of viable protozoa (Diamond, 1964). Early work showed that *B. bigemina* infected blood remained infective to cattle for almost 2 years using glycerol as the cryoprotectant and storing at -79 °C (Barnett, 1964; Dalgliesh, 1972). Dalgliesh (1972) demonstrated that dimethylsulfoxide (DMSO) effectively cryopreserved *B. bigemina* at -79 °C and later optimized the cooling rates for cryopreserving the parasite in DMSO at -196 °C (Dalgliesh and Mellors, 1974). Standfast and Jorgensen (1997) demonstrated that both DMSO and polyvinylpyrrolidone -40 (PVP-40) served equally well as cryoprotectants for *B. bovis* or *B. bigemina* infected blood but suggested that use of PVP-40 might be advantageous due in part to less toxicity to the parasite. The preferred method of cryopreserving cultured *Babesia* spp. is based on the work of Palmer et al. (1982). Polyvinylpyrrolidone (PVP) is the cryoprotectant of choice with a cooling rate of - 20 °C/min down to - 80 °C and then transfer to storage at -196 °C (Canning and Winger, 1987). PVP differs from glycerol or DMSO in function as a cryoprotectant by not diffusing through the plasma membrane of the parasite at the time of thawing, which often results in osmotic shock with the latter (Dalgliesh, 1972). The protocol of Palmer et al. (1982), where the cell pellet is mixed with an equal volume of freezing medium

consisting of 20% PVP-40 (polyvinylpyrrolidone-40) in Puck's saline glucose with extra glucose (w/v) to achieve a final concentration of 10% PVP-40, cooled at a rate of - 20 °C/min with final storage at -196 °C, has resulted in successful cryopreservation of numerous *Babesia* spp. (Holman et al., 1988; Holman et al., 1994; Hentrich et al., 1994; Jackson et al., 2001). To date, limited information is available regarding the viability of cryopreserved *Babesia* spp. following extended storage, despite its wide usage. In addition to validating the cryopreservation method for preserving viable cultured *Babesia*, the successful recovery of archived samples will provide early passage populations and allow genomic and/or antigenic evaluation of parasite populations over time. The two objectives of this study were to (1) identify an animal that was a suitable bovine blood donor for culturing bovine *Babesia* spp. *in vitro*, and (2) determine whether cryopreserved cultured parasites stored at - 196 °C for periods up to 30 years could be successfully returned to culture. In this study we investigated the recovery of both *B. bovis* and *B. bigemina* cryostocks and the suitability of four cattle as erythrocyte and serum donors.

Materials and Methods

Bovine blood donors

Blood was obtained from 4 cattle housed at the Animal and Plant Health Inspection Service, Veterinary Services, National Veterinary Services Laboratories, Ames, IA, using animal protocol APH-2016-537. The animals included a 2-year-old Angus steer (#913), weight 703 kg; 2 8-year-old Jersey steers (#58 and #61) and a 7-year-old Jersey steer (#1393), each weighing approximately 653 kg. The cattle were housed in an open concrete lot with access inside and bedded on corn stalk and straw. They were fed a total mixed ration of 9 kg corn silage, 5.7 kg grass hay, 1.8 kg soybean meal and 2.7 kg dry cattle pre-mix. Blood was collected via jugular

venipuncture into 7 ml BD Vacutainer tubes containing K3 EDTA and 10 ml BD Vacutainer serum tubes (Becton Dickinson Co., Franklin Lakes, NJ) at 2-week intervals and shipped on ice overnight to Texas A &M University, College Station, TX. On arrival, the whole anticoagulated blood was transferred to 15 ml centrifuge tubes and centrifuged for 30 min at 200× g at 4 °C to pellet the cells. The plasma and buffy layer were removed and the cell pellet washed 3 times by centrifugation with at least 5 volumes of RPMI-1640 (Lonza Walkersville, MD USA) with removal of the supernatant and residual buffy layer at each wash. The final erythrocyte pellet was resuspended in an equal volume of Puck's Saline Glucose (PSG) (Alfa Aesar, Word Hill, MA) plus extra glucose (20 g glucose/l) (PSG + G) with a final concentration of 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml Amphotericin-B (Antibiotic Antimycotic Solution; Mediatech, Inc., Manassas, VA) and stored refrigerated until use. The serum tubes were centrifuged after clot formation to separate the serum. The serum was dispensed into 20−40 ml aliquots and stored frozen at −20 °C for future use in media.

Babesia bovis *and* B. bigemina *cryostocks*

The *Babesia bovis* and *B. bigemina* cryostocks were MASP cultures previously established at Texas A &M University (P.J. Holman, unpublished data), using standard protocols (Erp et al., 1980; Vega et al.,1985a,b). The cultures were cryopreserved in 1986 and 1987 according to Palmer et al. (1982) except that the prepared cryostocks were placed in a −80 °C ethanol bath overnight before final storage in liquid nitrogen (−196 °C).

Babesia bigemina recovery after cryopreservation

Duplicate wells in 24-well culture plate each received 0.9 ml medium containing serum from animal #1393, #913, #61 or #58. Matching erythrocytes and serum, 0.1 ml of washed

bovine erythrocytes from animal #1393, #913, #61 or #58 was added to the medium in each pair of wells. The media consisted of HL-1™ Chemically Defined Serum-free Media (Lonza) with 20% adult bovine serum (from either #1393, #913, #61 or #58), 200 mM L-glutamine (Atlanta Biologicals, Lawrenceville GA), and 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml Amphotericin-B (Mediatech, Inc.). Three cryovials of Passage 95 cultured *B. bigemina* (~0.75 ml/vial, parasitemia ≥7%) cryopreserved June 19, 1987, were removed from liquid nitrogen storage and the contents thawed rapidly by Immediately swirling the vials in a 37 °C water bath. The contents of the vials were pooled and 250 μl added to each of the prepared wells to bring the total volume of each well to 1.25 ml. The plates were placed in a humidified modular incubator unit (Queue Systems, West Virginia, USA), the unit flushed with a gas mixture of 2% oxygen, 5% carbon dioxide, and 93% nitrogen at 2 lbs/sq inch pressure for 1 min and then incubated at 37 °C. Early passage cultured *B. bigemina* cryostocks from June 16, 1986, (6passages 2 and 3) and June 23, 1986, (passage 6) were thawed, pooled, dispensed into prepared wells of a 24-well plate, and incubated as above.

Babesia bovis recovery after cryopreservation

Two cryopreserved batches of the same *B. bovis* isolate were plated as described above, but only with #913 erythrocytes and medium containing #913 serum. Cultures were initiated in 2 wells of a 24-well culture plate from early passage *B. bovis* culture designated as HC and cryopreserved July 1, 1986. Similarly, cultures were initiated in 4 wells of a 24-well culture plate from early passage *B. bovis* culture designated as PF and also cryopreserved July 1, 1986. The plates were placed in a humidified modular incubator unit which was then flushed with the gas mixture of 2% oxygen, 5% carbon dioxide, and 93% nitrogen at 2 lbs/ sq. inch pressure for 1 min and incubated as above.

Culture maintenance and monitoring

Approximately 16 h after recovery, the medium overlying the settled cell layer was removed and a thin smear prepared from < 1 μl of the settled cells. The medium was replaced (approximately 800–900 μl) and the plate was returned to the incubator unit which was then flushed with the gas mixture and returned to the 37 °C incubator. The thin smear was air-dried, methanol fixed and again air-dried, then stained with Giemsa (Sigma Accustain) according to the manufacturer's recommendations. The stained smear was viewed by light microscopy at 1000× under oil immersion to monitor for the appearance of parasites. The recovered cultures were replenished daily with fresh medium in concordance with the RBC. For each well, 0.9 ml medium was removed without disturbing the cell layer and a thin smear made as above. Each well was then replenished with 0.9 ml of the appropriate medium. The smears were stained as above and examined daily to monitor parasite growth and to determine the percent parasitized erythrocytes (PPE). The PPE was determined from 1000 total erythrocytes counted with a manual electronic cell counter (Differential Counter Model 111, Fisher Scientific) at 1000X magnification under oil immersion light microscopy. The cultures were first subcultured when 7 parasitized erythrocytes per 1000X field were seen in more than one field in a Giemsastained smear. To subculture, the culture medium was replenished and the smear made as above, then the cultured cells were resuspended and0.25 ml transferred to a new well containing 0.9 ml media and 0.1 ml appropriate erythrocytes. Subsequent subcultures were similarly performed as the PPE reached ≥1%. As the cultures became reestablished and propagating so that they were subcultured at regular intervals, they were incubated in a humidified 5% carbon dioxide in air atmosphere.

Cryopreservation and resuscitation of B. bovis

Recovered culture HC *B. bovis* in the 3rd passage after recovery from liquid nitrogen storage was cryopreserved. The culture was centrifuged at 200× g to pellet the cells and the medium was removed. An equal volume of cold cryoprotectant medium (PSG + G with 20% PVP-40 (Sigma, St. Louis, MO)) was added to the cell pellet, the cells were gently resuspended and 0.25 ml was dispensed into each cryopreservation vial. The vial was placed in a - 80 °C ethanol bath overnight and then transferred to liquid nitrogen storage. Cryopreserved HC *B. bovis* was resuscitated from liquid nitrogen after 6 months in storage. The plate preparation and inoculation of 2 wells was as described above using donor #913 RBC and serum. The plate was placed in a modular incubator unit with a humidified atmosphere of 2% oxygen, 5% carbon dioxide, and 93% nitrogen as above. The cultures were maintained and monitored daily as above.

Results

Babesia bigemina was reestablished from both early passage and passage 95 cultures recovered from liquid nitrogen preservation after storage for nearly 30 and 29 years, respectively (Figs. 3.1 and 3.2). Piroplasms were first seen in #913 RBC in the early passage *B. bigemina* recovered culture on day 4 after recovery and on day 4 in the passage 95 culture. The parasites continued to thrive and were first passaged at a subculture ratio of 1:5 on day 17 for the early passage culture and day 15 for the passage 95 culture (Figs. 3.1-3.3). *B. bigemina* growth was maintained only in the culture wells with # 913 erythrocytes and #913 supplemented medium. Parasitized #58 RBC were seen in the passage 95 culture but were too few to determine the PPE. The parasites did not thrive and the culture was not passaged. No parasitized erythrocytes were observed in the cultures with #61 or #1393 serum and donor RBC. The recovered 95th passage parasites were subcultured as they reached a PPE of approximately 1% for the first month of

culture, after which the PPE was 2–3% when subcultured (Fig. 3.2). Subculture intervals varied from 2 to 5 days. The early passage *B. bigemina* was more robust and was subcultured as a PPE of 2–3% was reached during the first month (Fig. 3.1). Overall, the restored growth varied from a PPE of about 2– 4.6% (3.35 average) for the first 3 months of culture in the 2% oxygen, 5% carbon dioxide, and 93% nitrogen atmosphere. After 89 days, in passage 18, the cultures were moved to a humidified 5% CO2 in air atmosphere, where the PPE increased over two days to 14%. On day 102, at a PPE of 8.3, the cultures were cryopreserved.

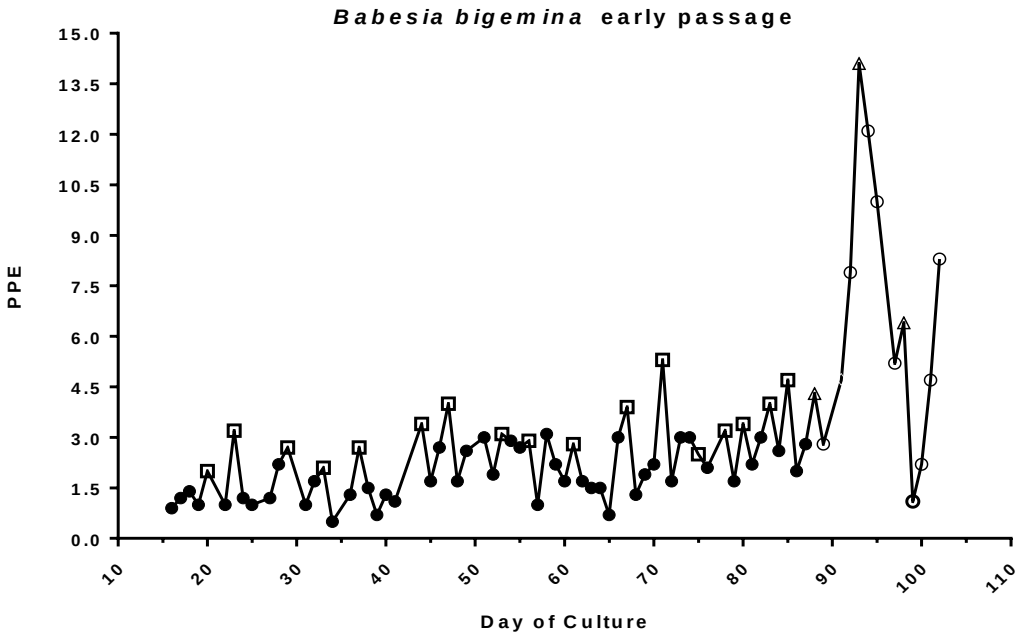

Fig. 3.1 Growth of early passage cultured *Babesia bigemina* recovered from cryopreservation. The percent parasitized erythrocytes (PPE) in the culture are shown on days between subcultures incubated in a humidified gas mixture of 2% O_2, 5% CO_2 and 93% N_2 atmosphere (●), PPE on day of subculture in the gas mixture (□), on days between subculture when incubated in a humidified 5% CO_2 in air atmosphere (○), and on the day of subculture in 5% CO_2 (Δ) (Reprinted with permission from Alhaboubi et al., 2017b).

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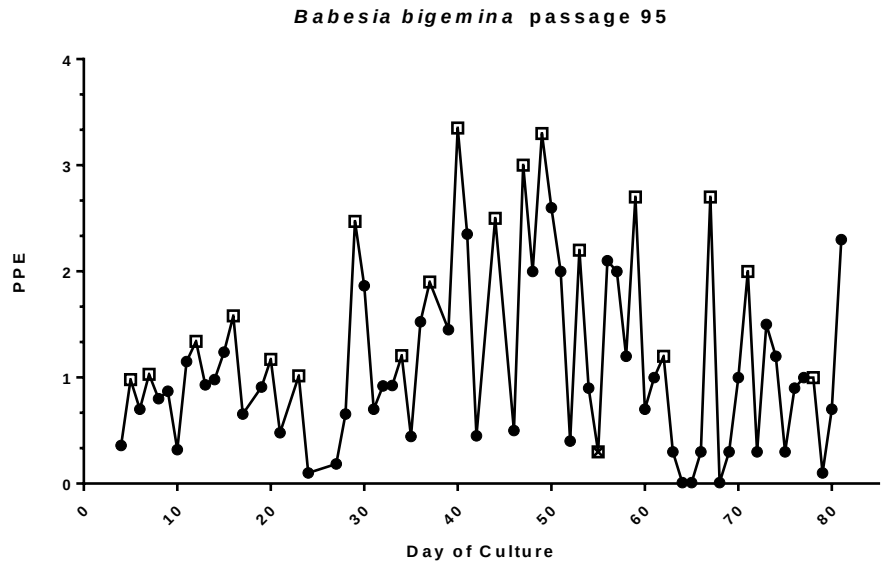

Fig. 3.2 Growth of passage 95 cultured *Babesia bigemina* recovered from cryopreservation. PPE between subcultures (●) and PPE on the day of subculture (□) **(**Reprinted with permission from Alhaboubi et al., 2017b).

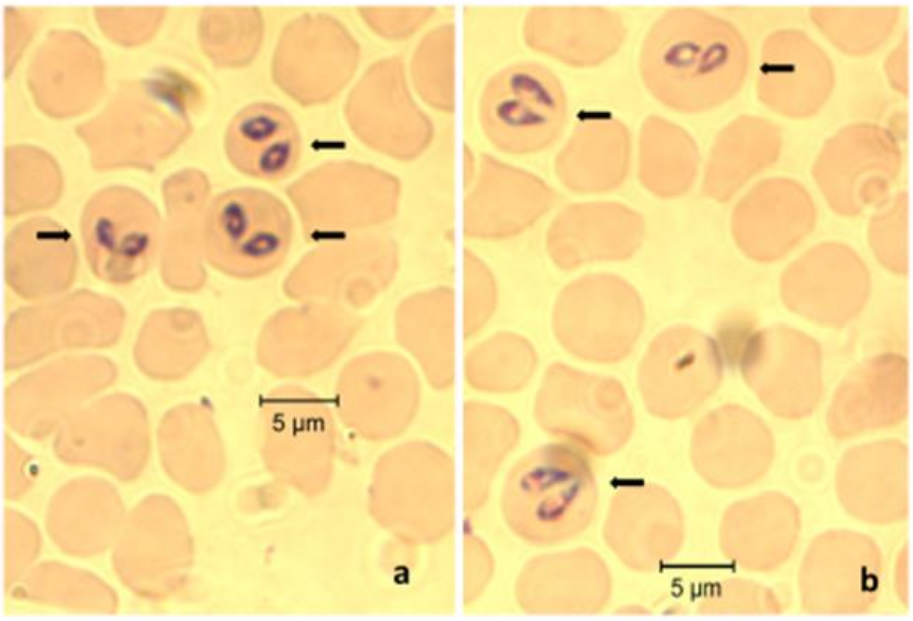

Fig. 3.3 Paired pyriforms (black arrows) of *Babesia bigemina* in culture passage 4 (a) and passage 6 (b) after recovery from cryopreservation. Giemsa stain, 1000X oil immersion **(**Reprinted with permission from Alhaboubi et al., 2017b).

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The two different batches of *B. bovis*, designated HC and PF, were successfully recovered after nearly 30 years of storage in liquid nitrogen. Intraerythrocytic parasites were first observed on day 4 for PF and on day 5 for HC (Figs. 3.4–3.6). The first subcultures were on day 13 for HC and day 18 for PF at a split ratio of 1:5. On day 19 the second subculture of HC was performed when the PPE was 4%. The PF culture propagation was slower initially so that the second subculture was not achieved until day 28 when the PPE was 4.5%. In general, the cultures were subcultured at 2–3 day intervals as the PPE reached 4–6%. Both the HC and PF cultures underwent parallel periods of unexplained lagging growth (days 28–33; days 37–41; days 61–65). The HC and PF cultures underwent 15 and 13 passages, respectively, in 74 days before they were terminated. The HC culture cryopreserved in the 3rd passage after recovery was resuscitated 6 months later. Intraerythrocytic parasites were first observed on day 4.

The first passage was on day 15 at a PPE of 3% (Fig.3. 7). The culture was maintained for 52 days through 12 passages which were done as the PPE reached ≥3%, except for the 7th passage when the PPE was < 2% (Fig. 3.7). A lag occurred between the 7th and 8th passages, after which the growth rate resumed.

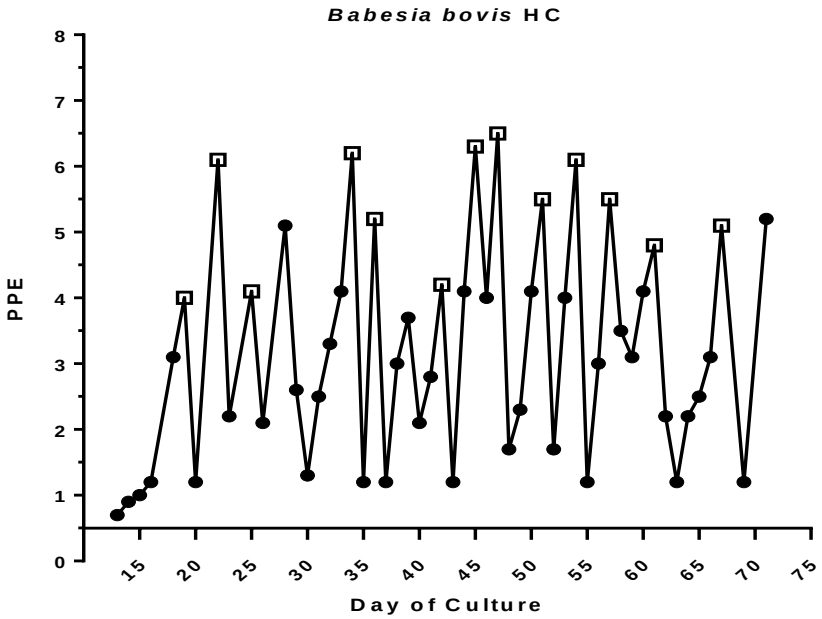

Fig. 3.4 Growth of *Babesia bovis* HC recovered from cryopreservation. PPE between subcultures (●) and PPE on the day of subculture (□) **(**Reprinted with permission from Alhaboubi et al., 2017b).

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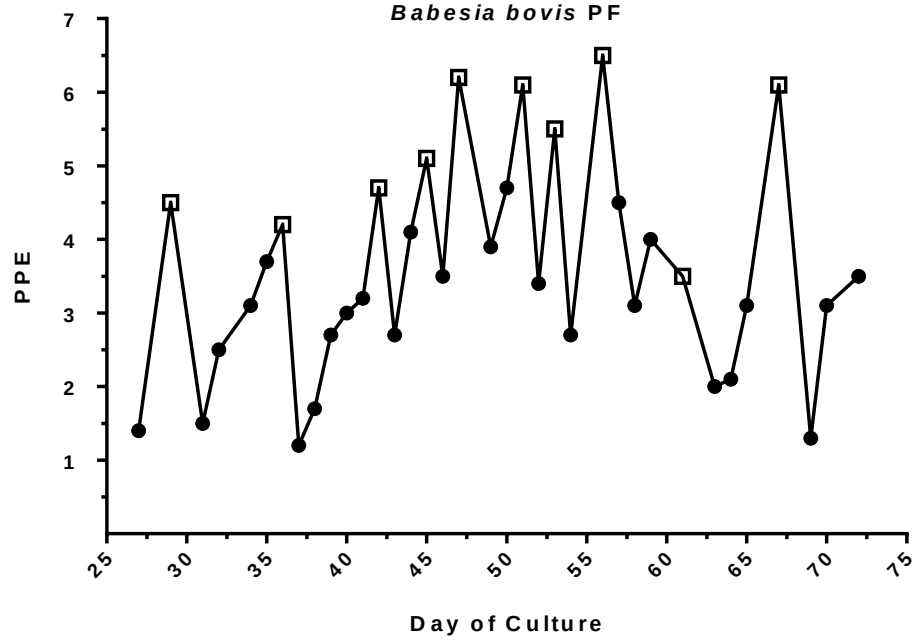

Fig. 3.5 Growth of *Babesia bovis* PF recovered from cryopreservation. PPE between subcultures (●) and PPE on the day of subculture (□) **(**Reprinted with permission from Alhaboubi et al., 2017b).

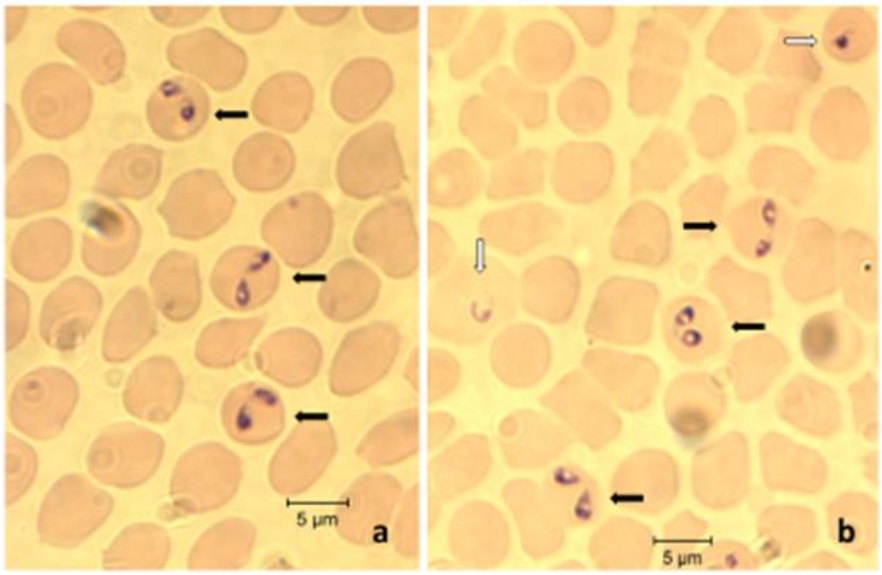

Fig. 3.6 *Babesia bovis* after recovery from cryopreservation. Paired pyriforms (black arrows) and singles (white arrows) of *Babesia bovis* in culture passage 2 (a) and passage 10 (b) are shown. Giemsa stain, 1000X oil immersion **(**Reprinted with permission from Alhaboubi et al., 2017b).

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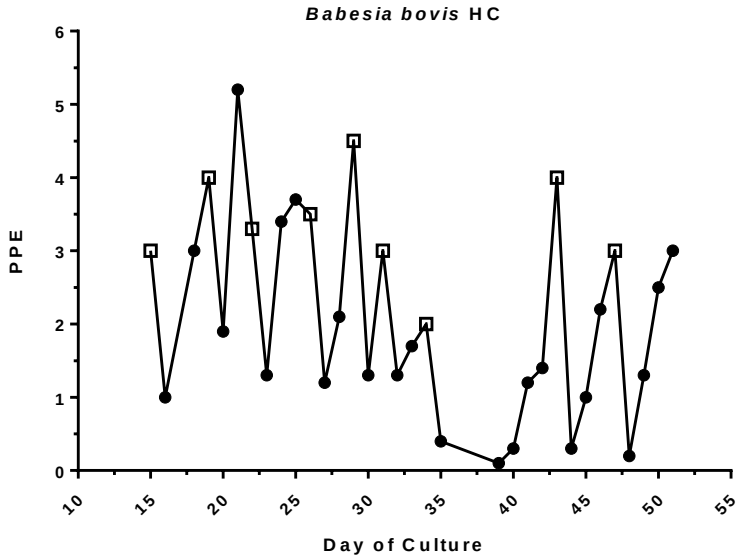

Fig. 3.7 Resuscitation of *Babesia bovis* culture frozen in the 3rd passage after recovery and stored for 6 months in liquid nitrogen. PPE (●) between subcultures and PPE at subculture (□) **(**Reprinted with permission from Alhaboubi et al., 2017b).

Discussion

The advantages of cryopreserving cultured *Babesia* spp. stocks are well-recognized, but there is a paucity of information regarding how long these stocks will remain viable under extended storage. There are reports of blood stabilates remaining infective for animals as long as 19 years of storage at -196 °C (Zweygarth and Lopez-Rebollar, 2000), but similar successful return of *Babesia* spp. to culture after lengthy periods of cryopreservation to our knowledge has not been reported. The current study shows that after nearly 30 years in liquid nitrogen storage, cultured *B. bovis* and *B. bigemina* were successfully recovered using the MASP system. The suitability of four donor cattle for the *in vitro* propagation of *B. bigemina* was examined in this study. Successful culture of both *B. bovis* and *B. bigemina* is highly dependent upon the animal donor providing erythrocytes and the serum component for the medium (Canning and Winger, 1987). Of the four animals compared in this study, only one supported the parasites in the erythrocytic stage culture. In this case, the animal was a 2-year-old Angus steer, whereas erythrocytes and serum from the three Jersey steers tested failed to support *B. bigemina in vitro*. However, bovine Babesia cultures by one of the authors (PJH) have been successfully propagated using erythrocytes and serum from both steers and cows, and of a variety of breeds including Angus X Brahman, Angus, Hereford, Angus X Hereford, Texas Longhorn, and Holstein (unpublished results). Thus, it could be argued that breed and/ or gender are not the critical factors. However, while there is in- formation regarding breed resistance to the tick vector and/or clinical babesiosis in vivo, i.e. *Bos indicus* breeds frequently experience milder clinical signs to primary *B. bovis* infections than *Bos taurus* breeds (Löhr, 1973; Callow, 1984; Bock et al., 1995; Bock et al., 1997; Bock et al., 1999) to our knowledge there are no studies regarding this phenomenon *in vitro*. In *vivo* this phenomenon is thought to be a result of the

evolutionary relationship between *Bos indicus* cattle, the tick vector, and *Babesia* spp. (Dalgliesh, 1993). Certainly Jersey cattle are susceptible to bovine babesiosis (Zahid et al., 2005; Velusamy et al., 2014). Age of the blood donor animal is an important critical factor to successful *in vitro* culture of *B. bovis* and *B. bigemina* (Levy et al., 1982; Canning and Winger, 1987). Calves experience no or mild clinical signs upon exposure to *B. bovis* or *B. bigemina* in *vivo*. It has been shown that cal erythrocytes and serum are unable to support continuous propagation of *B. bovis in vitro* (Levy et al., 1982). The inhibitory effect is antibody independent, is attributed to one or more unidentified dialyzable serum factors, and was present in all calves' donors tested (Levy et al., 1982). Studies indicate that the composition of the erythrocyte membrane may influence propagation of *B. bovis in vitro* (Okubo et al., 2007; Takabatake et al., 2007). Both cholesterol and sialic acid content affected the ability of *B. bovis* to invade the erythrocyte *in vitro* and the cholesterol content also affected the subsequent growth of the cultured parasites (Okubo et al., 2007; Takabatake et al., 2007). It should be pointed out that in the current work the growth medium consisting of the defined medium HL-1™ supplemented with 20% normal adult bovine serum successfully supported the initiation and maintenance of the cultures upon recovery from liquid nitrogen storage even though the cryopreserved *Babesia* spp. were originally cultured in a different medium. The use of HL-1™ medium was in- troduced over 20 years ago with the successful culture of *Babesia (Theileria) equi* (Holman et al., 1994), and since that time, it has gained acceptance as a reliable medium for culture of a number of *Babesia* spp. (reviewed by Schuster, 2002). However, to our knowledge, this is the first report of successfully introducing *Babesia* spp. resuscitated from frozen storage to culture in a medium different from the one in which they were maintained prior to cryopreservation. For the cryostocks dating back to 1986 and 1987, the cultures were originally propagated in M-199 with

Earl's salts supplemented with 40% normal adult bovine serum (Holman et al., 1993b). Thus, even though the parasites were reintroduced to culture under conditions unlike those when cryopre- served, they recovered and reestablished *in vitro*.

In conclusion, this study shows that cryopreserved *B. bovis* and *B. bigemina* stored nearly three decades in liquid nitrogen can be successfully recovered in the MASP system and also confirms previous observations that selection of a suitable bovine donor of erythrocytes and serum is critical to the success of the culture.

CHAPTER IV

GENOTYPING *BABESIA BOVIS* AND *BABESIA BIGEMINA* ISOLATES FROM PUERTO RICO AND MEXICO

Introduction

In parasite diagnosis, it is important to determine the parasite species, subspecies and genotype causing disease as the virulence, prognosis, and response to anti-parasitic drugs may vary among different strains or genotypes. In particular, diagnosis of *Babesia* infection is often based on morphological characterization including the size and the appearance of intra-erythrocytic forms in peripheral blood (Levine, 1988). Although finding the parasite during examination of the blood smears is the considered gold standard of identification methods, when the animals present with low parasitemia, morphological techniques may not be sensitive enough to detect infection, much less allow identification of the species (Böse et al., 1995). An Enzyme-Linked Immunosorbent Assay (ELISA) was developed as serodiagnostic application and is known to be more sensitive than microscopic diagnosis, particularly, in the case of animals with chronically low parasitemia. However, chronically infected animals might have antibodies that unpredictably cross-react against other *Babesia* species (Kuttler, 1981; Callow, 1984; Böse and Peymann, 1994). On the other hand, Polymerase Chain Reaction (PCR) and nested PCR targeting different genetic markers often provide practical tools to detect and distinguish infections with various *Babesia* spp. and are known to be 100 times more sensitive than microscopic examination (Saiki et al., 1988; Fahrimal et al., 1992; Persing et al., 1992; Figueroa et al., 1992, 1993). These molecular techniques constitute more specific and sensitive methods for accurate diagnosis and subsequent appropriate treatment and are being widely adopted as

alternative tools for detecting and identifying different pathogens (Reddy et al., 1991; Birkenheuer et al., 2003; Duarte et al., 2008).

Ribosomal DNA analysis is used for many epidemiological and phylogenetic studies of piroplasms (Kjemtrup et al., 2000; Altay, et al., 2005; Holman et al., 2002). Part of the ribosomal DNA transcriptional unit is the small subunit (SSU), or the 18S, ribosomal RNA (*18S rRNA*) gene. This gene is commonly used as a taxonomic marker and in phylogenetic studies (Woese et al., 1990; Zahler et al., 2000; Aktas et al., 2007). The *18S rRNA* gene is a reliable candidate because it is present in high copy number, which increases the sensitivity of detection. The ribosomal RNA in most eukaryotes is highly conserved and is organized in transcriptional units in tandem and is composed of the 18S, 5.8S, and 28S (large subunit, LSU) rRNA genes (Long and Dawid, 1980; Gutell and Fox, 1988; Kibe et al., 1994). Between the 18S and 5.8S and between the 5.8S and 28S genes are intergenic transcribed spacer regions (ITS1 and ITS2, respectively) (Dalrymple, 1990). The slow evolving sequence of the rRNA genes in living organisms is very useful to evaluate ancient evolutionary events. In addition, conservation of these genes has allowed the construction of many universal primers, which assist in sequencing efforts for newly studied groups (Hillis and Dixon, 1991), and have been useful as wide-spectrum diagnostic tools.

While the rRNA genes are highly conserved, the ITS regions are not function-constrained as are the rRNA genes but are divergent and distinctive regions subjected to higher evolutionary rates leading to greater variability in both nucleotide sequence and length (Long and Dawid, 1980; Ferrer et al., 2001). Thus, the ITS regions are distinguished and different among closely related organisms (Lott et al., 1993; Williams et al., 1995; Lott et al., 1998). Thus, in order to differentiate between piroplasm species, and to discriminate within a species, the ITS regions are

more valuable than the rRNA genes. They allow discrete phylogenetic separation of closely related species, isolates, and the recognition of new species and subspecies (Zahler et al., 1998; Collins and Allsopp, 1999; Fazaeli et al., 2000; Dubey et al., 2001).

The members of the *Babesia* complex have a life cycle that includes the asexual intraerythrocytic stages in the bovine host (such as trophozoites and merozoites), while the sexual stages, such as ookinete formation, occur within the vector tick. Thus, each developmental stage will require different gene expression profiles depending upon the metabolic needs and environmental conditions (Fang and McCutchan, 2002).

Based on sequence analysis of the *18S rRNA* gene and ITS-2 region, three distinct rRNA transcriptional units, named A, B, and C, were identified in *B. bovis* (Laughery et al., 2009). The analysis determined nucleotide polymorphisms and sequence insertions distinguishing the three rRNA gene coding units. A differential comparison of rRNA gene transcription showed that *B. bovis* parasites preferentially expressed transcriptional unit B under *in vitro* culture conditions. It is suggested that the absence of variability in immune pressure, the presence of nutrients, and a consistent incubation temperature *in vitro* influenced expression of this rRNA coding unit. Further, Laughery et al. (2009) found that all three coding unit transcripts, A, B, and C, were co-expressed in *B. bovis* from the bovine host and from the vector tick (Laughery et al., 2009).

Babesia bovis glycosylphosphatidylinositol (GPI) anchor proteins, including the Variable Merozoite Surface Antigens (VMSA) family, are proteins expressed on the surface of the merozoite and/or sporozoite. Studies showed that antibodies against at least five proteins belonging to this family inhibit parasite penetration into the host erythrocyte (Hines et al., 1989; Hines et al., 1992; Florin-Christensen et al., 2002).

The VMSA genes encode a family of immunostimulatory proteins that are expressed on the merozoite surface and contain immunodominant B and CD4+ T cell epitopes (Brown et al., 1998; Hines et al., 1992). The *B. bovis* strain T2Bo genome contains five genes encoding VMSA, including *msa-1* and four copies of *msa-2*. The genes reside on chromosome 1, with the four *msa-2* copies arranged tandemly in a head-to-tail fashion and *msa-1* located 5 kbp upstream from the *msa-2* genes (Brayton et al., 2007). Genome analysis revealed similar gene placement for related Mexico strains of *B. bovis* (Florin-Christensen et al., 2002; Lau, 2009; Ferreri et al., 2012).

The VMSA are important in the pathogenesis of babesiosis because of their role in parasite recognition, attachment and invasion of the host erythrocyte (Jack and Ward, 1981; Hines et al., 1989; Florin-Christensen et al., 2002; Mosqueda, et al., 2002). Therefore, VMSA are potential target antigens for a protective immune response and are considered to be good vaccine candidates. However, VMSA antigenic polymorphism poses a challenge and may allow the parasite to effectively evade the host immune response when *B. bovis* VMSA is used as a vaccine (Katzer et al., 1994; Palmer and McElwain, 1995; Brown and Palmer, 1999). This antigenic polymorphism is demonstrated by radioimmunoassay, immunofluorescence assay, and immunoblotting using monoclonal antibodies and post-infection sera of a number of *B. bovis* geographical isolates (Texas, Mexico, Australia and Israel), which identified multiple epitopes exposed on the surface of the parasite (Goff et al., 1988; Reduker et al., 1989; Palmer et al., 1991). Thus, the differences that exist among geographically diverse strains may lead to a complete lack of immunologic cross-reactivity (Hines et al., 1995a; Suarez et al., 2000). However, phylogenetic studies revealed that these genes could be novel molecular markers for epidemiological investigations and determining geographical distribution relationships among

B. bovis isolates (Genis et al., 2008, 2009; Borgonio et al., 2008; Altangerel et al., 2012). Therefore, the variation seen among the VMSA may lend itself to use as a genotyping marker.

One of the genes that is expressed in both *B. bovis* sporozoites and merozoites is *msa2b* (Florin-Christensen et al., 2002). The gene has an open reading frame of about 864 bp and codes for the Merozoite Suface Antigene 2b (MSA-2b) protein of approximately 83 kDa. MSA-2b co-expresses with MSA-2c and/or MSA-2a1 and MSA-2a2 to facilitate host cell invasion. The MSA-2b gene marker has been investigated in *B. bovis* phylogenetic and genotyping studies, invariably focused on the geographical genetic diversity of *B. bovis* MSA genes in bovine hosts in endemic countries (LeRoith et al., 2005; Genis et al., 2008; Genis et al., 2009; Altangerel et al., 2012; Nagano et al., 2013; Sivakumar et al., 2013). The hypothesis for this study is the *18S rRNA* gene, rRNA ITS1-ITS2, and MSA-2b are reliable markers for genotyping *B. bovis*.

Specific objectives

The specific objectives of this study are:

1. To genotype different populations of *B. bovis* using the chromosomally encoded *18S rRNA* gene, rRNA ITS1 and ITS2 intergenic regions, and MSA-2b gene.

2. To analyze genetic differences in the three molecular markers among the isolates in this study and those in the NCBI GenBank® database.

Materials and Methods

Mexican Babesia *isolates DNA extraction*

DNAs were extracted from 8 archived cultured *Babesia* spp. samples parasites following the manufacturer's protocol (FlexiGene DNA Extraction kit, Qiagen, Redwood City, CA, USA).

The extracted DNA concentration was determined by (NanoDropND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and adjusted to approximately 100 ng/µl

Puerto Rico Babesia spp. *DNA*

Forty one *Babesia* spp. DNA samples were provided for this study by the Cattle Fever Tick Research Laboratory (CFTRL) in Mission, Texas from cattle blood samples acquired in Puerto Rico during 2014-2015. The DNA samples were prescreened by scientists at the research laboratory for the presence of *Babesia bovis* or *Babesia bigemina* DNA by a PCR assay that detects regions of the *18S rRNA* gene (Guerrero et al., 2007). Samples positive for either or both species were provided for this study. The DNA samples were quantified prior to use as above.

PCR assays for 18S rRNA *gene, ITS regions, and* msa-2b

The nearly full-length *18S rRNA* gene was amplified by PCR in a primary assay with universal primers A and B (Table 4.1). The amplification reaction was performed according to the manufacturer's instructions (EX polymerase, TAKARA BIO INC., Japan) in a final volume of 25 µl containing ~100 ng of DNA. Positive control (positive reference plasmid DNAs for *B. bovis* and *B. bigemina 18S rRNA* gene were derived from MASP cultures previously established at Texas A&M University, Patricia J. Holman, unpublished) and negative control (no DNA) reactions were included. The amplification profile was initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min followed by hold at 4 °C. A nested PCR was used for all isolates with the reaction volume, reagents, and amplification profile the same as above, except that the template DNA consisted of 1 µl of the primary PCR

product and primers AN50 and BN1700 (Table 4.1) were used to amplify an approximately 1600 bp fragment.

The genomic region spanning the rRNA ITS1, *5.8S* gene, and ITS2 was amplified by PCR using *B. bovis* 528F and LSUR primers (Table 4.1). Positive control (positive reference plasmid DNAs for *B. bovis* and *B. bigemina* were derived from MASP cultures previously established at Texas A&M University, Patricia J. Holman, unpublished) and negative control (no DNA) reactions were included. The amplification profile was an initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min followed by hold at 4 °C. Nested PCR was used for all isolates with the reaction volume, reagents, and amplification profile the same as above, except that 1 µl of the primary PCR product and a 1:5 dilution of the product served as template DNA and primers Bbov1600F and LSURN (Table 4.1) were used to yield a fragment of approximately 700 bp.

An approximately 820 bp fragment within the *msa-2b* locus was PCR amplified using the forward primer MSA-2a1/2a2/2bF (Sivakumar et al., 2013) and reverse primer PRmsa-2R (slight modification of Sivakumar et al., 2013) (Table 4.1). The amplification reaction was performed according to the manufacturer's instructions (EX polymerase, TAKARA BIO INC., Japan) in a final volume of 25 µl containing ~100 ng of DNA. Positive (plasmid DNA from pre-cloned and sequenced *B. bovis msa-2b*) and negative (no DNA) control reactions were included. The amplification profile was an initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 7 min followed by hold at 4 °C. A nested PCR was used for all isolates with the reaction volume, reagents, and amplification profile as above, except that 1 µl

of the primary PCR product and a 1:5 dilution of this same product served as template DNA. In the nested PCR, primers PRmsa-2bF (slight modification of Sivakumar et al., 2013) and MSA-R (Sivakumar et al., 2013) (Table 4.1) were used to generate an amplicon of the *msa-2b* variable region of approximately 680 bp.

Table 4.1 Primers for PCR and sequencing the *18S rRNA* gene, rRNA ITS region, and *msa-2b*.

Use	Primer	Sequence 5′→3′	Annealing	Reference
Primary 18s	A, forward	ACCTGGTTGATCCTGCCAG	60 ºC	Sogin, 1990
Primary 18s	B, reverse	GATCCTTCTGCAGGTTCACCTAC	60 ºC	Sogin, 1990
Nested 18s	AN, forward	GCTTGTCTTAAAGATTAAGCCATGC	60 ºC	Schoelkopf et al., 2005
Nested 18s	BN, forward	CGACTTCTCCTTCCTTTAAGTGATAAG	60 ºC	Schoelkopf et al., 2005
Primary ITS	528EXTF, forward	CGGTAATTCCAGCTCCAATAGC	56 ºC	Holman et al., 2011
Primary ITS	BbovLSUR, reverse	CTTGTCTGCCGCTTAGTTATAGC	56 ºC	Holman et al., 2011
Nested ITS	Bbov1600F, forward	TGCGCGATCCGTCG	56 ºC	Holman et al., 2011
Nested ITS	BbovLSURN, reverse	GGATAGCCTCGTACATCTCAGG	56 ºC	Holman et al., 2011
Primary msa-2R	MSA-2a1/2a2/2bF,forward	ATGATCGGGAAAATCTTCTTGTTAA	55 ºC	Sivakumar et al., 2013
Primary msa-2R	MSA-R, Reverse	TTAAAATGCAGAGAGAACGAAGTA	55 ºC	Sivakumar et al., 2013
Nested msa-2b	PRmsa2bF, Forward	AAGTCGATATGCCTTCCGATAACTC*	55 ºC	Sivakumar et al., 2013*
Nested msa-2b	PRmsa-2b, Reverse	AATGCAGAGAGAACGAAGTAG**	55 ºC	Sivakumar et al., 2013**
Sequencing	M13F	GTAAAACGACGGCCA	50 ºC	N/A***
Sequencing	M13R	CAGGAAACAGCTATGAC	50 ºC	N/A

* Slight modification of Sivakumar et al., 2013: removal of the GC nucleotides from the 3' end.
**Modification of PRmsa-2b (Sivakumar et al., 2013): removal of AA nucleotides from the 5' prime and TA nucleotides from the 3'end.
***N/A, not applicable

Cloning and sequencing

PCR products were resolved by electrophoresis alongside a 200-bp marker (BioDL200 BioFlux, Bulldog Bio, Portsmouth, NH, USA) through a 1% agarose gel and stained with ethidium bromide (0.5 μg/ml) to visualize the bands using UV transillumination (ChemiDoc Imaging System, Carlsbad, CA, USA). PCR products visualized as single bands were directly ligated into a plasmid and cloned as below. The appropriate-sized band was gel purified from products with multiple bands using the Montage DNA Gel Extraction Kit (Millipore Sigma, Darmstadt, Germany) prior to cloning.

Within 24 h of amplification, amplicons of ~1600 bp for the 18S RNA gene, ~700 bp for the ITS region and ~680 bp for *msa-2b* were directly ligated into the PCR™ 2.1-TOPO™ TA cloning vector and incorporated into chemically competent *Escherichia coli* cells using heat shock transformation protocols following manufacturer's instructions (TOP 10 One Shot®; Invitrogen, Grand Island, NY, USA). Transformed colonies were randomly chosen for colony PCR to verify the presence of inserts of the correct size. Verified colonies were expanded in overnight cultures to generate plasmid DNA to sequence the cloned PCR fragments for confirmation and analysis. Plasmid DNA was extracted from the cultures using conventional plasmid DNA purification kits (Plasmid DNA Minipreps Kit, Promega Inc., Madison, MI, USA). The plasmid DNA samples were adjusted to a concentration of approximately 100 ng/μl and submitted for automated bi-directional Sanger sequencing (Eurofines Inc, Louisville, KY, USA) using universal M13 sequencing primers (Table 4.1) or the amplification primers. The resulting sequences were analyzed using MacVector© Assembler version 16.0.8 (MacVector Inc., Apex, NC, USA) and trimmed to remove ambiguous or unreadable data. Chromatogram-based contiguous sequences were generated and similarities to gene sequences in the GenBank®

database determined using the NCBI Basic Local Alignment Tool (BLAST) (Altschul et al., 1990). Pairwise comparisons between the sequences were calculated using the program in the MacVector© Assembler software.

Nucleotide sequence accession numbers

All newly obtained sequences were submitted to the NCBI GenBank® database and assigned accession numbers (Appendix A). The new 33 accessions for the *B. bovis* Puerto Rico *18S rRNA* gene were assigned MH045741-MH045772 and MH050386, while the 12 cultured *B. bovis* isolates were assigned MH046902-MH046913. The Puerto Rico *B. bigemina* were assigned the numbers MH047814-MH047819 and MH050387, and the cultured Virgin Island *B. bigemina* isolate MH050356.

The rRNA ITS sequences were submitted to NCBI GenBank® database and assigned accession numbers (Appendix A). The Puerto Rico *Babesia bovis* isolates were assigned MH050902-MH050904, MH050906-MH050908, and MH050911-MH050925. The cultured *B. bigemina* 1987 was assigned MH050926.

The *msa-2b* sequences were submitted NCBI GenBank® database and assigned accession numbers (Appendix A). The Puerto Rico *B. bovis* isolates were assigned MH064400- MH064402 and MH064414-MH064413, while the cultured *B. bovis* isolates were assigned MH064403-MH064413.

Phylogenetic analysis

Alignments of the obtained 18S rDNA and rRNA ITS1-*5.8S* gene-ITS2 sequences with corresponding sequences from the GenBank® database (Tables 4.2 and 4.3, respectively) were made using the ClustalW default setting in MacVector© Assembler version 16.0.8. The 18S

rDNA spanned from position 95 to 1543 in *B. bovis* (based on GenBank® accession number L19077). The rRNA ITS region was delineated at the 3' end based on HQ264129 for both *Babesia* spp. while EF458270 and EF458243 were used for delineating the ITS-2 5' end of *B. bovis* and *B. bigemina*, respectively. The unweighted pair-group method with arithmetic mean (UPGMA) Sokal and Michener, 1958 using the Kimura two-parameter model (Kimura, 1980) was used to generate trees from the resulting alignments, with 1000 bootstrap replications for clustering robustness (MacVector© Assembler version 16.0.8).

Alignments of the deduced MSA-2b amino acid (AA) sequences of the obtained *msa-2b* cloned sequences with corresponding sequences from the GenBank® database (Table 4.4) were made using the ClustalW default setting in MacVector© Assembler version 16.0.8. All MSA-2b sequences were trimmed to include AA position 73 to 266 based on the sequence for *B. bovis*, GenBank® accession number LC004311. The UPGMA tree was predicted from the alignment with 1000 bootstrap repetitions, random tie breaking, and Poisson-correction setting using MacVector© Assembler version 16.0.8. *B. bovis* MSA-1 (AB612247) served as the outgroup.

The MSA-2b deduced amino acid sequences from PR6 Clone 1 (MH064416) and Mexico HUASTECA Clone 2 (MH064410) were selected randomly as representative for each group and analyzed for diversity between the Puerto Rico and culture sample sequences and to predict the B-Cell antibody epitope. The methods for predicting continuous antibody epitope from protein sequences using an online Bepipred Linear Epitope Prediction tool (IEDB Analysis Resources, http://tools.iedb.org) and the parameters for antigenicity were verified using the protein analysis tool in MacVector© Assembler version 16.0.8. All PR and culture cloned sequences were evaluated in MSA-2b deduced amino acid sequence alignments for the presence of the epitopes identified in PR6 Clone 1 and in Mexico HUASTECA Clone 2.

Table 4.2 GenBank® *Babesia* spp. *18S rRNA* gene sequences included in the alignment.

Accession number	Species	Strain/Isolate	Country	Submitter/Reference
KM046917	*B. bigemina*	Swiss_6	Switzerland	Liu et al., 2016
KP745628	*B. bovis*	Trbrt36	Turkey	Aktas et al., 2015
KP745623	*B. bigemina*	Trkoz10	Turkey	Aktas et al., 2015
KU206297	*B. bigemina*	MT25	Uganda	Byaruhanga et al., 2015*
KU206296	*B. bigemina*	MT26	Uganda	Byaruhanga et al., 2015*
KU206295	*B. bigemina*	KT4	Uganda	Byaruhanga et al., 2015*
KP710223	*B. bovis*	ZJK15	China	Zou et al., 2015*
KF928959	*B. bovis*	Bareilly	India	Mandal et al., 2014
JQ437260	*B. bovis*	8284 Dixie	Australia,	Dawood et al., 2013
JQ437262	*B. bovis*	H81	Australia	Dawood et al., 2013
JX495403	*B. bovis*	boLushi	China	Tian et al., 2013
JX495402	*B. bigemina*	boLushi	China	Tian et al., 2013
JQ723013	*B. bovis*		China	Du et al., 2012*
HQ840960	*B. bigemina*	563	China	He L et al., 2012
HQ264111	*B. boivs*	USDA Ames	USA	Holman et al., 2011
HQ264112	*B. boivs*	USDA Ames	USA	Holman et al., 2011
FJ426364	*B. boivs*	BRC01	Brazil	Criado-Fornelio et al., 2009
FJ426361	*B. bigemina*	BRC02	Brazil	Criado-Fornelio et al., 2009
EF458198	*B. bigemina*	B_bi09	Brazil	Vogl and Zahler, 2007*
EF458204	*B. bigemina*	B_bi17	Mexico	Vogl and Zahler, 2007*
EF458205	*B. bigemina*	B_bi18	Puerto Rico	Vogl and Zahler, 2007*
DQ785311	*B. bigemina*	Spain_1		Buling et al., 2007
DQ287954	*B. ovis*		Goat 2	Criado et al., 2006
AY603398	*B. bovis*	Shannxian	China	Luo et al., 2005
AY603402	*b. bigemina*	Kunming	China	Luo et al., 2005
AY150059	*B. bovis*		Portugal	Criado-Fornelio et al., 2003
L19077	*B. bovis*	Vaccine S	South Africa	Allsopp et al., 1994
L19078	*B. bovis*	Vaccine S	South Africa	Allsopp et al.,1994
M87566	*B. bovis*	Samford	Australia	Ellis et al., 1992
X59607	*B. bigemina*		Mexico	Reddy et al.,1991

*Unpublished

Table 4.3 GenBank® *Babesia* spp. rRNA ITS region sequences included in the alignment.

Accession number	Species	Isolate	Country	Submitter/Reference
KF928960	*B.bovis*	Bareilly	India	Mandal et al., 2014
HQ264121	*B.bovis*	Merida cl.3	Mexico	Holman et al., 2011
HQ264122	*B.bovis*	Merida cl.4	Mexico	Holman et al., 2011
HQ264128	*B.bovis*	H8cl.10	TX, USA	Holman et al., 2011
HQ264129**	*B.bovis*	H8cl.14	TX, USA	Holman et al., 2011
HM538271	*B.bigemina*	Clone-6	China: Suizhou	Wang et al., 2010
EF458270**	*B.bovis*	B_bo18	Mexico	Vogl et al., 2007*
EF458274	*B.bovis*	B_bo01	Turkey	Vogl et al., 2007*
EF458275	*B.bovis*	B_bo02	Argentina	Vogl et al., 2007*
EF458278	*B.bovis*	B_bo03	Australia	Vogl et al., 2007*
EF458294	*B.bovis*	B_bo11	Australia	Vogl et al., 2007*
EF458297	*B.bovis*	B_bo13	Mexico	Vogl et al., 2007*
EF458298	*B.bovis*	B_bo14	Uruguay	Vogl et al., 2007*
EF458299	*B.bovis*	B_bo15	South Africa	Vogl et al., 2007*
EF458300	*B.bovis*	B_bo16	USA	Vogl et al., 2007*
EF458302	*B.bovis*	B_bo17	USA	Vogl et al., 2007*
EF458243**	*B.bigemina*	B_bi07	Brazil	Vogl et al., 2007*
EF547926	*B.bovis*		China Lushi	Liu et al., 2007*

*Unpublished

***Babesia* sp. isolates used for delineation of the ITS1-ITS2 rRNA region

Table 4.4 GenBank® *Babesia bovis msa-2b* sequences included in the alignment.

Accession number	Isolate	Country	Submitter/Reference
KU522560	Tc 43.2	Brazil	Matos et al., 2016*
APY23928	RJ-42.3F-C10	Brazil	Matos et al., 2016*
LC004332	BU25.2	Viet Nam:Hue	Yokoyama et al., 2015
LC004311	CA78.1	Viet Nam:Hue	Yokoyama et al., 2015
LC004312	CA7.2	Viet Nam:Hue	Yokoyama et al., 2015
LC004322	CA124	Viet Nam:Hue	Yokoyama et al., 2015
LC004309	CA63.1	Viet Nam:Hue	Yokoyama et al., 2015
AB819785	P180	Philippines: Badian	Tattiyapong et al., 2014
AB819779	P14	Philippines: Alcoy	Tattiyapong et al., 2014
KJ152551	GON	Israel	Molad et al., 2014
AK441805		Texas	Yamagishi et al.,2014
AB787620	p75.1	Sri Lanka	Sivakumar et al., 2013
AB787628	AM24	Sri Lanka: Ampara	Sivakumar et al., 2013
AB612261	No. BI-9	Mongolia	Altangerel et al., 2012
AB612247	BI-12	Mongolia	Altangerel et al., 2012
AB745696	Bbo-ca-N9	Thailand: North	Simking et al., 2012
FJ422802	RAD	Mexico City	Dominguez et al., 2008
EF644337		Mexico	Genis et al., 2008
FJ597639	Nuevo Leon	Mexico	Genis et al., 2008
EF644341	Guerrero	Mexico	Genis et al., 2008
EF644339	Chiapas	Mexico	Genis et al., 2008
EF644340	Quintana	Mexico	Genis et al., 2008
EF644348	Jalisco	Mexico	Genis et al., 2008
EF644347	Nayarit	Mexico	Genis et al., 2008
DQ173947	F3	Ausralia	Berens et al., 2005
ABA06441	GO6	Australia	Berens et al., 2005
ABA06440	F40	Australia	Berens et al., 2005
DQ173955	L	Australia	Berens et al., 2005
DQ173948	F35	Australia	Berens et al., 2005
AY052541	RIA	Argentina	Florin-Christensen et al., 2002

*Unpublished

Results

18S rRNA *gene*

Genomic DNA from 41 *Babesia*-infected blood samples from cattle in Puerto Rico and 8 *Babesia* spp. isolates from previously archived blood or culture samples was used in this study (Tables 4.5-4.7). The *18S rRNA* gene was amplified by PCR from 26 of the Puerto Rico (PR) samples and 6 of the archived samples, for a total of 54 cloned sequences. Of these, nearly full-length *18S rRNA* genes were successfully amplified with primers A and B only from 4 culture

samples. Nested PCR with AN and BN primers produced *18S rRNA* gene sequences for the remaining 28 samples. The products were cloned and sequenced (Tables 4.5-4.7). The nearly full-length *B. bovis* and *B. bigemina 18S rRNA* gene sequences were determined from overlapping contiguous sequences and varied in length from ~1600 base pairs (bp) with A/B primers and ~1500 bp with the AN/BN primers.

Table 4.5 *Babesia bovis 18S rRNA* gene, ITS region and *msa-2b* cloned sequences obtained from culture isolates in this study**.**

Isolate Details				Number of Clones		
Isolate ID	**Origin**	**Isolate**	**Year**	***18S rRNA***	**ITS**	***msa-2b***
Ames	(AKA) USA IA	archived culture	1986	3	0	3
Huasteca (Hust)	Mexico	archived culture	1986	2	0	2
Bbov1986	Mexico	archived culture	1986	3	0	1
BbovC275	Mexico	archived culture	1980s	2	0	3
BbovC123	Mexico	archived culture	1991	2	0	2
Total		5		12	0	11

The obtained *18S rRNA* gene sequences aligned closely within the conserved regions with minor differences observed in the variable regions. The percent identity among the 33 PR *B. bovis* isolate cloned sequences was 92.3% to 100% (with conserved identities = 1312 bp) (Appendix C). The identity score among the 12 cloned sequences of the Mexico *B. bovis* isolates ranged from 97.7% to 99.99% (with conserved identities = 1440 bp) (Appendix C). The identity score among the 8 cloned sequences of the Puerto Rico and the VI (Virgin Island) culture *B. bigemina* isolates ranged from 99.1% to 99.98% (with conserved identities =1457 bp) (Appendix C).

Table 4.6 *Babesia bovis 18S rRNA* gene, ITS region and *msa-2b* cloned sequences obtained from the Puerto Rico samples in this study.

Isolate Identification	Collection Year	Number of Clones		
		18S rRNA	**ITS**	***msa-2b***
Puerto Rico 1	2014	2	2	0
Puerto Rico 2	2014	3	2	2
Puerto Rico 4	2014	2	1	2
Puerto Rico 5	2014	2	1	1
Puerto Rico 6	2014	3	0	2
Puerto Rico 7	2014	3	0	0
Puerto Rico 8	2014	2	1	1
Puerto Rico 9	2014	1	1	3
Puerto Rico 17	2014	2	1	0
Puerto Rico 19	2014	1	1	1
Puerto Rico 21	2014	2	1	0
Puerto Rico 22	2014	0	0	2
Puerto Rico 23	2014	0	0	1
Puerto Rico 24	2014	1	0	1
Puerto Rico 31	2014	1	1	1
Puerto Rico 32	2014	1	1	1
Puerto Rico 33	2014	0	2	0
Puerto Rico 34	2014	2	1	0
Puerto Rico 35	2014	1	1	0
Puerto Rico y-500	2016	1	0	0
Puerto Rico 890	2016	1	0	0
Puerto Rico 1086	2016	2	0	1
Total 22		33	14	17

*The *18S rRNA* BLAST result identifies this cloned sequence as *B. bigemina*

Table 4.7 *Babesia bigemina* 18S rDNA and ITS cloned sequences obtained from Puerto Rico and archived VI culture samples.

Isolate Identification	Origin	Collection Year	ITS
BBIG_VI 1988	Virgin Islands	Archived 1980s	0
Puerto Rico 11	Puerto Rico	2014	0
Puerto Rico 12	Puerto Rico	2014	1*
Puerto Rico 25	Puerto Rico	2014	1*
Puerto Rico 28	Puerto Rico	2014	0
Puerto Rico 36	Puerto Rico	2014	0
Puerto Rico 37	Puerto Rico	2014	1*
Puerto Rico 38	Puerto Rico	2014	0
Total 8			3

*The BLAST result, identifies the ITS cloned sequence as *B. bovis*

The UPGMA cladogram tree was constructed with *Babesia* spp. *18S rRNA* gene cloned sequences of approximately ~1500 bp (Fig. 4.1). The tree depicts the clear distinction of *B. bovis* and *B. bigemina* (and *B. ovis)* 18S rDNA sequences separated into two well-supported major clades (bootstrap value 99). The *B. bovis* cloned sequences from Puerto Rico and Mexico branches into two groups within one clade, with the exception of *B. bovis* PR17 clone 2, which branches separately. The first *B. bovis* group consists of 16 Puerto Rico cloned sequences that split into eight PR sequences clustering with sequences from Portugal and Turkey with moderate support (bootstrap value 76), while the other eight PR sequences clustered together in one group with sub-branches highly supported (bootstrap values of 86 or 99). Within the second group of the *B. bovis* clade the sequences obtained in this study are found in two branches holding two cloned sequences each (one with two PR clones, and one with a PR clone and a Hust clone), and in three larger clusters. In one cluster, the *B. bovis* vaccine strain S (L19078) is branched with cultured samples (Ames and BbovC123) from this study, one PR clone branches with two isolates from China (China and ZJK), three PR clones branch together, and three PR clones branch independently within the group. In another cluster, there are two branches with Hust,

Bbov1986 and BbovC275 culture clones together with sequences from India, the vaccine strain S and Mo7 Mexican isolate, and two PR sequences occupying the other branch. A third cluster within this *B. bovis* group holds four PR sequences.

The second major clade holds *B. bigemina* sequences and *B. ovis* branched separately with a strong bootstrap value (96). The *B. bigemina* group shows the PR and cultured *B. bigemina* from the Virgin Islands interspersed with sequences from China, Uganda, Mexico, Brazil, Switzerland, Spain and Puerto Rico.

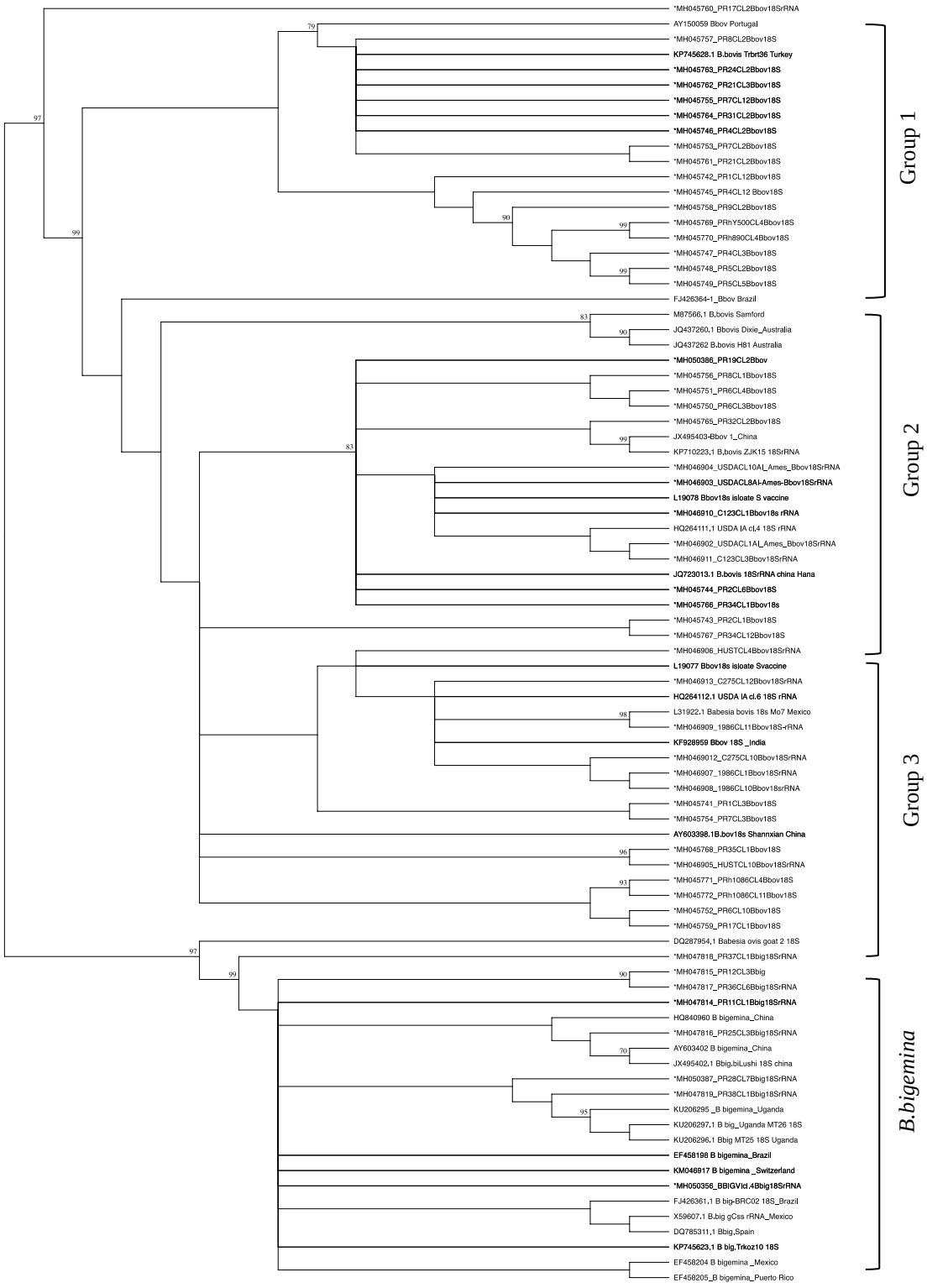

Fig. 4.1 UPGMA tree for the *18S rRNA* gene sequences of *B. bovis* and *B. bigemina*. The numbers on the branches represent the percentage of bootstrap values (1000 replicates).

Ribosomal RNA ITS region

The ITS1-ITS2 rRNA region was amplified by nested PCR from a total of 17 samples (16 Puerto Rico, and 1 *B. bigemina*) out of 49 *Babesia* spp. DNA samples (Tables 4.5-4.7). The amplicons were cloned and 21 clones were sequenced (Tables 4.5-4.7). The full length ITS region was ~ 650 base pairs (bp) (Appendix B). The rRNA ITS sequences aligned with the conserved regions with differences observed in the variable regions. The percent sequence identity among the 21 clones from the Puerto Rico *B. bovis* isolates was 69.3% to 100% (with conserved identities = 367 bp) (Appendix C).

The *5.8S rRNA* gene is located at position 336 through 481, between the ITS1 and ITS2 regions. The *5.8S* gene had less sequence variation compared to I1 and ITS2. Among the Puerto Rico clones the identity scores range from 95.9-100%.

The UPGMA tree of the ITS region (Fig. 4.2) was derived from ~ 630 bp of the *B. bovis* ITS sequences. The tree shows two major clades, one is a strongly supported (bootstrap value 99) out-group branch holding *B. bigemina* sequences (culture and from China) and the other, also strongly supported (bootstrap value 99), holding *B. bovis* sequences. Within the *B. bovis* clade, the largest cluster holds 20 PR interspersed with sequences from GenBank® that branch into 7 groups (Fig. 4.2). Groups 1 and 2 are comprised of only PR sequences, whereas Groups 3-7 hold both PR and sequences from other geographical isolates. Two smaller strongly supported clusters hold sequences from Australia and South Africa isolates (bootstrap value 99), and PR and Turkey (bootstrap value 99).

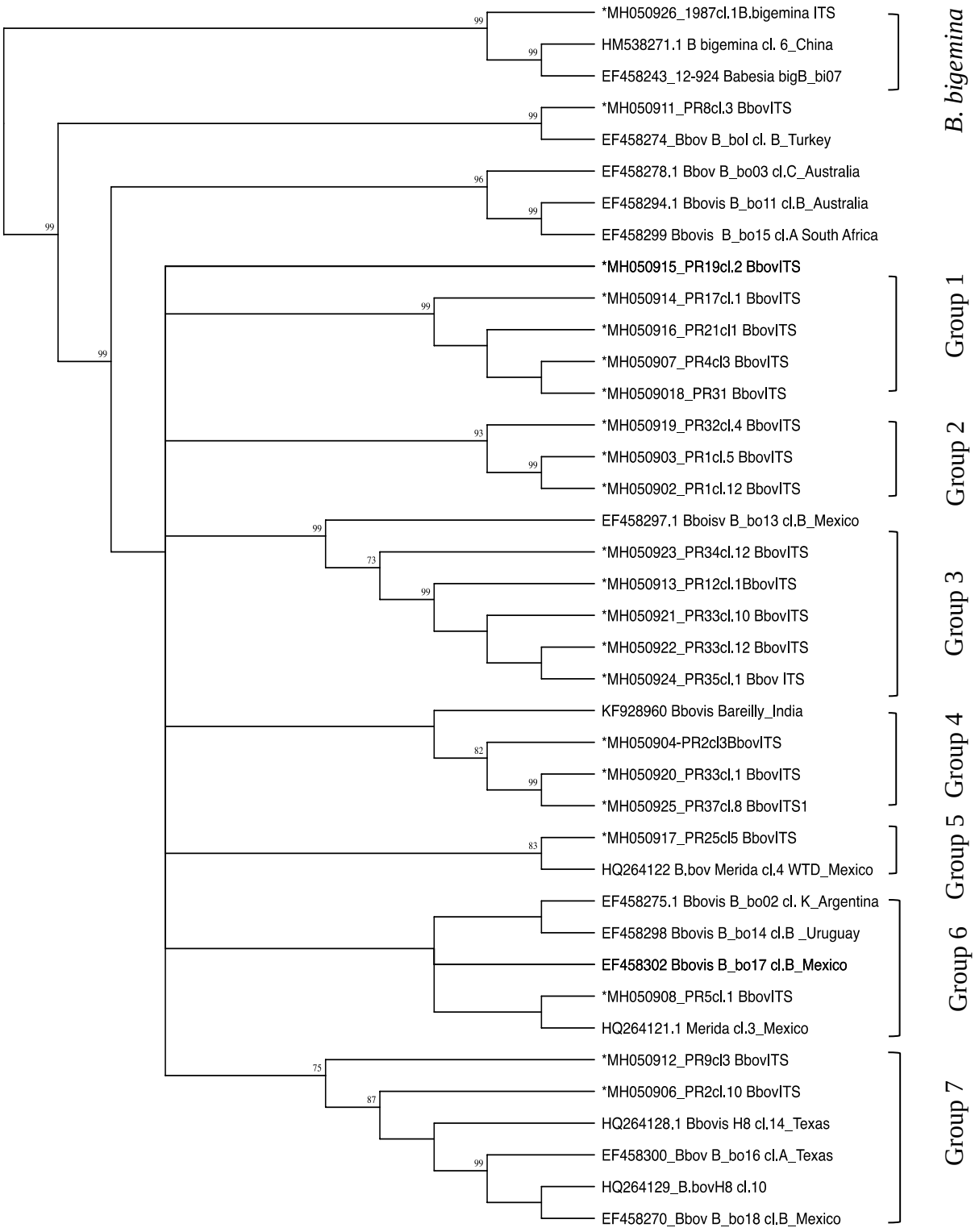

Fig. 4.2 Phylogenetic tree constructed by the UPGMA method for the ITS rRNA sequences of *B. bovis* and *B. bigemina*. The UPGMA tree used 1000 bootstrap replicates. The numbers on the branches represent the percentage bootstrap value**.**

Merozoite surface antigene 2b

In total 47 DNA samples, of which 24 samples were confirmed *B. bovis* by *18S rRNA* gene sequence, were subjected to PCR amplification for the *msa-2b* gene. Thirteen of the 24 confirmed *B. bovis* samples yielded products in nested PCR resulting in 30 cloned sequences (Tables 4.5 and 4.6). No *msa-2b* amplification occurred in DNA samples not confirmed by *18S rRNA* gene analysis to be *B. bovis*. Sequence analysis of the cloned amplicons showed that the length of the partial coding region sequence varies from 645 to 733 bp between the culture isolates, and 630 to 717 bp among the Puerto Rico isolates.

Within the Puerto Rico samples, sequence identity scores among the deduced MSA-2b amino acid sequences isolates were diverse and ranged from 41.2% to 100% while they ranged from 45.4% to 100% between the archived samples (Appendix C).

Both PR and culture samples share multiple B-cell epitope sequences (Table 4.7). The B- cell epitopes predicted in PR6 Clone 1 and Mexico (Hust) Clone 2 sequences were identified in all cloned sequences, but variability was found as shown in Table 4.7. In addition to the epitopes shown in Table 4.7, a 47 amino acid long peptide which was quite variable in sequence was identified by the analysis.

The phylogenetic tree was constructed with UPGMA method from the MSA-2b deduced amino acid sequences of the PR and the archived samples *msa-2b* sequences, using the AB612247 MSA-1 gene from Mongolia as an outgroup (Fig. 4.3). The topology of the tree shows two major clades with high support (bootstrap value 99). One clade holds two groups, one with four cloned sequences of two Puerto Rico isolates that are clustered with sequences from Mexico, Argentina and Philippines, while the second holds two culture sequences (Hust. and Ames) clustered with sequences from Mexico and Texas.

The other clade is comprised of two major clusters (Fig. 4.3). Cluster 1 branches into two groups. One group forms two branches with one holding six PR sequences and the other holding six cultured *B. bovis* sequences with one from Mongolia. The other group holds a branch of four PR sequences and a branch of Australia and Viet Nam sequences. Cluster 2 intersperses PR sequences among sequences from Australia, Thailand, Viet Nam, Brazil and Sri Lanka (Fig. 4.3).

The cloned PR sequences showed four main sequence types and one outlier sequence whereas the culture clones showed 3 main sequence types and one outlier sequence. There was no duplication in sequence types between PR and culture clones.

Table 4.8 Consensus B-cell epitopes predicted from Puerto Rico and culture Mexico MSA-2b sequences.

Puerto Rico		**Mexico (culture)**	
Epitopes	Conserved aa/total	Epitopes	Conserved aa/total
KVPF(K/E)TSL	3/8	KVPFKTSL	3/8
LQELDQ	2/6	YQDTDE	2/6
NDNPPHMLAN	7/10	NDNPPHLLTN	7/10
CKEDxEVKD	3/9	SAKDyz†VK	3/8

x=A,G,V, S, or T
y=A,G, or S
†z= N, E, or D

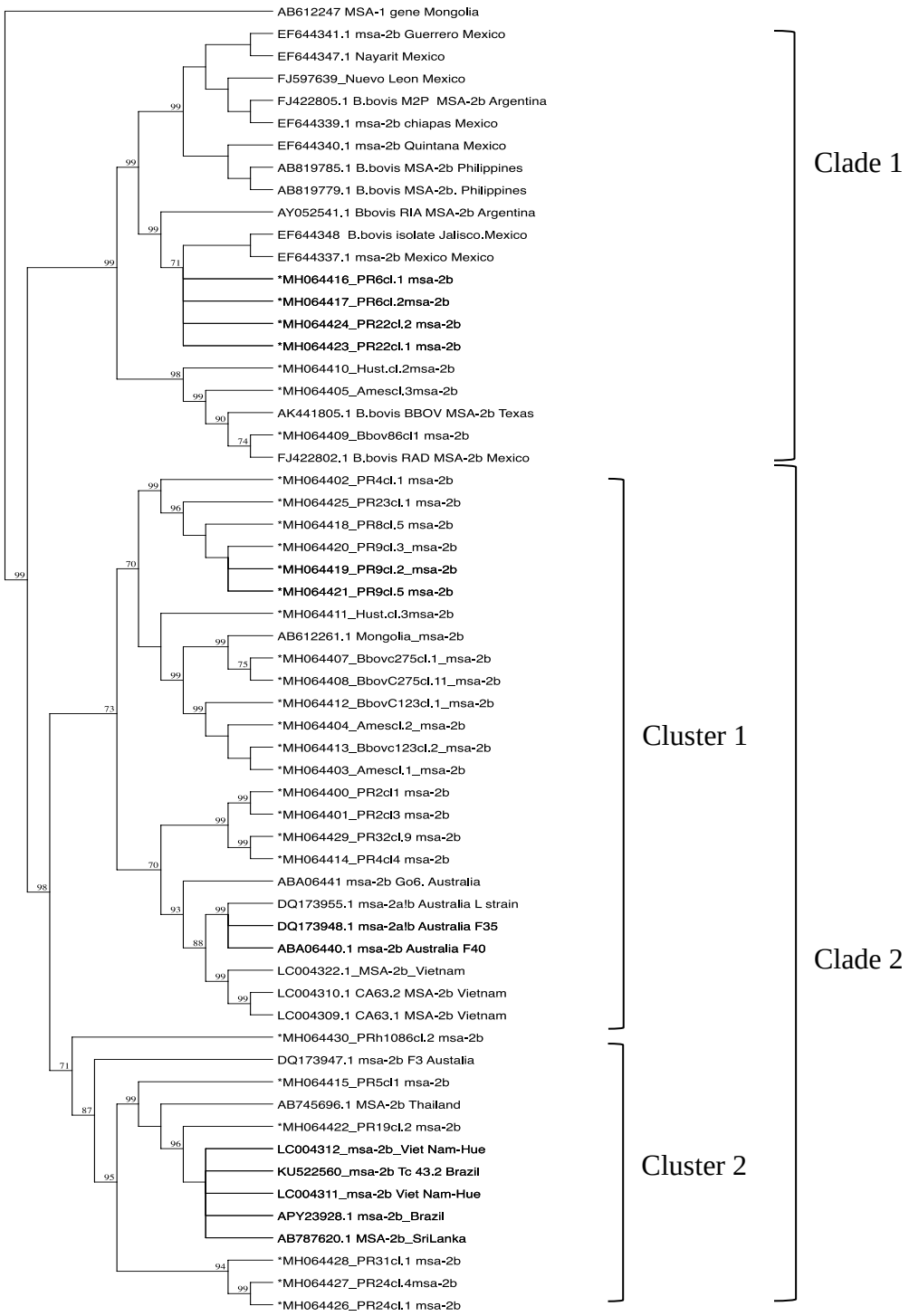

Fig. 4.3 UPGMA method for the deduced amino acids of the *msa-2b* genes of *Babesia bovis*. The UPGMA tree used 1000 bootstrap replicates. The number represents the percentage of bootstrap values.

Discussion

The current study investigated genotyping *B. bovis* isolates from Puerto Rico using three markers, the *18S rRNA* gene, rRNA ITS1 and ITS2 intergenic regions, and *msa-2b* gene. Several cultured *B. bovis* isolates from Mexico were included in these analyses. Furthermore, in the current study, the *18S rRNA* gene and rRNA ITS regions were compared among *B. bigemina* isolates from Puerto Rico, Mexico and the Virgin Islands.

The current *B. bovis* and *B. bigemina 18S rRNA* gene assessment was based on cloning and sequence analysis. The nucleotide sequences obtained from *B. bovis* isolates from Mexico and Puerto Rico were similar to each other with slight differences, which has also been reported in previous studies (Holman et al., 2002; Birkenheuer et al., 2004). For example, the full gene sequences for *B. bovis* from Portugal revealed less than 95% identity with corresponding sequences that were reported in the GenBank® database (Criado-Fornelio et al., 2003). Although the *18S rRNA* gene is highly conserved and has been widely used for the identification of many piroplasms, both the current results and previous reports include discordant nucleotide sequences between the *18S rRNA* gene of piroplasm isolates, as well as among cloned sequences from a single isolate (Allsopp et al., 1994; Calder et al., 1996; Kim et al., 2007; Ramos et al., 2010). One possible explanation for these differences in *Babesia* spp. is the existence of rRNA A, B, and C transcriptional units, which are reported for both *B. bovis* and *B. bigemina* (Laughery et al., 2009; Reddy et al., 1991). The results of the current study also show differences among the cloned cultured *B. bovis* and *B. bigemina 18S rRNA* gene sequences.

The phylogenetic tree generated from the *Babesia* spp. 18S rDNA sequences obtained in this study and by others proposed two main groups of the *Babesia* spp. including a major clade of *B. bovis* and a second clade representing *B. bigemina* and *B. ovis* as an outgroup. The

divergence of *B. bovis* and *B. bigemina* was described earlier in previous phylogenetic studies (Ellis et al., 1992). The topology of the current tree shows clustering of a large number of the Puerto Rico *B. bovis* sequences with the Portugal (AY150059) and the Turkey (KP745628) sequences.

A limitation of the current study was the number of *B. bovis* and *B. bigemina* sequences from different geographic regions available in the GenBank® database. Furthermore, many of the available sequences were too short, which restricted the use of other cloned sequences from certain geographical areas. In this study, we preferred to include only the longer sequences and to exclude the shorter obtained sequences, because short gene sequences are not reliable for drawing phylogenetic conclusions. The results of the latter approach may be misleading, as previously noted by Caccio and collaborators (2002).

Another limitation relates to the sequence conservation due to functional constraints of the *18S rRNA* gene so that genetic variation may not be sufficient to lead to distinguishing between similar species and furthermore at the strain level.

Due to the aforementioned limitations, sequence variation of the rRNA ITS1-5.8S-ITS2 regions in *B. bovis* from within an endemic area of Puerto Rico and from culture isolates was examined. Because the ITS regions are not under the structural limitations imposed on the ribosomal genes, these regions may provide finer distinction among closely related species (Collins and Allsopp, 1999) and were employed to further examine the relationships among the Puerto Rico *B. bovis* isolates. Cloned sequences spanning this region were examined in the current study. Again, there was a shortage of corresponding comparative full-length sequence data for this marker from different geographic areas, being limited to available GenBank® submission data of Vogl et al. (2007) and Holman et al. (2011). The ITS UPGMA phylogenetic

tree showed strong bootstrap support for four clades. The cloned *B. bovis* sequences obtained in this study were separated into seven groups within one large clade. Bootstrap support was strong for most branches holding Puerto Rico isolates. The ITS1 and ITS2 regions had more variation compared to the *18S* and the *5.8S* gene regions. Most of the differences observed in the ITS cloned sequences could be attributed to single-nucleotide polymorphisms (SNP).

Genes associated with antigenic variation have been reported, even between other similar protozoal parasites, such as *Theileria* spp. (Kibe et al., 1994; Schoelkopf et al., 2005; Aktas et al., 2007; Holman et al., 2011) and *Babesia canis* (Zahler et al., 1998). In this current study of *B. bovis msa-2b* cloned sequences and phylogenetic analysis, at least four different *msa-2b* genotypes are present in the Puerto Rico sample population, and two genotypes are present among the culture samples of Mexican origin. Genetic diversity of merozoite surface antigens in *B. bovis* was previously detected from Sri Lanka cattle (Sivakumar et al., 2013). Genetic diversity is postulated to be the major way of generating polymorphisms in this gene family, and most likely occurs during *B. bovis* stages in the tick vector (Hoffman and Stoffel 1993; Jasmer et al., 1992).

The comparison of MSA-2b proteins from *B. bovis* isolates from infected cattle in Puerto Rico reveals distinctions among the cloned sequences of an individual isolate, which agrees with previous observations. Genis et al. (2008) reported genes encoding exceptionally polymorphic, surface expressed proteins with consensus sequence identity for MSA-2b proteins of 73.5%. In this study, we extended phylogenetic analyses to include a number of MSA-2b sequences from *B. bovis* isolates endemic in Sri Lanka, Vietnam-Hue, Brazil and Thailand, and additional isolates from Europe, Asia, and Africa, to better correlate with previous studies as recommended by Genis and others (2008).

According to the current MSA-2b phylogenetic analysis, the Puerto Rico isolates form their own clusters separate from the Mexican *B. bovis* isolate clusters and separate from clusters holding other geographic isolates with stronger bootstrap support than previously reported (Genis et al., 2008). Overall, the phylogenetic analysis supports the genotypic diversity of merozoite surface antigens of *B. bovis* within an endemic population (Lau et al., 2010) and, in some cases, geographic regions.

It is postulated that a *B. bovis* vaccine breakthrough isolate would represent extensive variation within the MSA-2b, which is, in fact, demonstrated by the recovery of Australia *B. bovis* with various MSA-2b sequences (strains F3, G06, F35 and F40) (Berens et al., 2005). In this study, many of the cloned Puerto Rico MSA-2b protein sequences are found in the same cluster phylogenetically as Australia vaccine breakthrough strains G06, F35 and F40 (Fig. 4.3, Cluster 1). Moreover, MSA-2b sequences obtained in this study, PR2cl.1, PR2cl.2, PR32cl.9 and PR4cl4 are found on a branch with weak bootstrap support in the same group within Cluster 1 as Australia G06, F35 and F40, indicating relatedness between the PR and Australia MSA-2b proteins. This suggests that it is possible that PR MSA-2b may be derived from Australian isolates as group of origin.

B. bovis msa-2 has two conserved regions, the amino-terminal region encoding the leader sequence and carboxy-terminal region encoding the glycosylphosphatidylinositol (GPI) anchor signal in the central, extramembranous region. Adjacent to the GPI, the carboxy-terminus contains proline-rich regions, which are repeated and form the region of highest polymorphisms, known as the hypervariable region (HVR). The HVR is one of the most hydrophilic regions of these molecules (Berens et al., 2005). The 3′-UTR is conserved in all the sequences obtained in this study and contains a U-rich region and polyadenylation signal (AATAA), which are known

to be conserved in all MSA family members. It is suggested that this region plays an important role in *B. bovis* VMSA gene expression (Florin-Christensen et al., 2002; Hines et al., 1992).

A decade ago, for practical purposes, a PCR–RFLP (restriction fragment length polymorphism) assay was implemented in which *EcoRI*, *HindIII* or *PstI* restriction enzymes defined *B. bovis msa-2b* fragment polymorphism profiles to discriminate *msa-2b* genotypes at the isolate/strain level (Genis et al., 2009). In the current study, DNA sequencing methods were used to discriminate among the *B. bovis msa-2b* genotypes present in Puerto Rico. Although direct sequencing from the PCR product is considered a less costly and time-consuming option, it is known that cloning the PCR product into a plasmid vector prior to sequencing in order to obtain multiple cloning sequences will help avoid missing any spurious low copy DNA amplicons (Carr et al., 2007). Cloning offers the capability of detecting polymorphisms at a low frequency, which otherwise could be masked by the sequence in abundance and missed with direct sequencing (Ruecker et al., 2011). Therefore, this study utilized cloning of the *B. bovis msa-2b*.

Since the current study utilized a *Taq* DNA polymerase to amplify *B. bovis msa-2b* it is possible that some of the observed polymorphisms may have been incorporated during amplification. It is difficult to determine whether artifact insertion occurs during PCR. This is especially true in the case of amplification from a highly heterogeneous pool of molecules, such as possible coinfection with multiple strains of a *Babesia* sp., which may lead to ambiguous sequences. In the current study, it appears that cloning was useful in lowering the occurrence of ambiguous, unreadable sequences, and the unreadable and short sequences that did occur were necessarily excluded from analysis. Unfortunately, this resulted in decreased useable data.

Although the UPGMA method is simple and less popular in use than the neighbor joining (NJ), both algorithms generate phylogenetic trees based on a distance-matrix method. In contrast with NJ, UPGMA employs sequential clustering to construct a rooted phylogenetic tree. A distance matrix of the analyzed data is calculated from a multiple alignment and the clustering is measured in average linkage analyses. The most reliable tree is required for revealing the most probable evolutionary relationships among organisms. Thus, in this study we opted to use the UPGMA method to construct our phylogenetic trees from the sequences generated.

It is suggested that recombination might be a common event among the *msa-2b* gene sequences and could contribute to the genetic diversity of these genes (Berens et al., 2005; Simuunza et al., 2011). In this study, an analysis of the genetic diversity among the *B. bovis msa-2b* gene predicted amino acid sequences show patterns in the isolates from cattle in Puerto Rico. We hypothesized that MSA-2b sequence diversity would be minimal within a geographical host population, however, we found that the MSA-2b sequences were found in two clades phylogenetically, and that within the second clade the sequences branched into numerous groups. Similarly, diversity was found in the 18S rDNA and the rRNA ITS sequences as well. To our knowledge, this is the first investigation of *msa-2b* in *B. bovis* from Puerto Rico combined with other molecular markers. Heterogeneity in 18S rDNA, ITS1-ITS2 region and MSA-2b sequences of the Puerto Rico *B. bovis* isolates suggest the possibility that *B. bovis* was re-introduced in infected cattle that were brought in from different geographic regions.

There is a need to sequence more *Babesia* genomes to allow better characterization of strains circulating in the environment. In the future, the developing technology of next generation sequencing (NGS), which will become more widely available and can be employed to establish a sensitive genotyping system based on testing multi-locus typing, would be highly

advantageous in genotyping apicomplexans. The new NGS technology (e.g., PacBio and Illumina) will allow advances in this field, and lead to more robust methodologies such as genotyping by sequencing to evaluate the genetic diversity of pathogenic *Babesia* species affecting cattle and other livestock, as well as those affecting humans and companion animals. Furthermore, it may lead to a better understanding of the genetics underlying *Babesia* spp. that circulate in enzootic cycles without causing disease.

CHAPTER V

CONCLUSION

Biological phylogenies were historically based on phenotypic characteristics of organisms, but more recently molecular phylogenies based on genotypic differences, including the encoded protein sequences, are employed and are being widely used for molecular taxonomy, population genetics, and phylogenetic analyses. The phylum Apicomplexa is a parasite group that shares several morphological characteristics and has been regrouped due to the new molecular techniques. The Apicomplexa phylum includes Haematozoa which include the Piroplasmida (piroplasms) and Haemosporidia (haemosporidia), while the Coccidea include the Adeleina (haemogregarines) and Eimeriina (haemococcidia). Chapter I presents a literature review of two intraerythrocytic apicomplexans, namely *Babesia* in the Piroplasmida and *Haemogregarina* in the Adeleina.

Chapter II focuses on morphological and molecular characterization of a *Haemogregarina* sp. in an alligator snapping turtle in Texas. The molecular characterization of the *Haemogregarina* sp. was based on *18S rRNA* gene sequence analysis as previously reported. Five cloned sequences of this gene in the *Haemogregarina* sp. from the alligator snapping turtle were obtained. Variation was found among the sequenced clones and none was identical to each other. Nonetheless, the clones were more like one another than to other reported *18S rRNA* gene sequences for haemogregarines. The highest sequence identity (98% - 99%) was to an unnamed *Haemogregarina* sp. found in the Caspian freshwater turtle *Mauremys caspica* in Fars Province in southern Iran (Rakhshandehroo et al., 2016), however, only a short 18S rDNA sequence (774 bp) is available for this parasite (GenBank® accession no. KR006985). *Haemogregarina* sp. of an alligator snapping turtle might be expected to be more closely related to *Haemogregarina*

balli found in the common snapping turtle *Chelydra serpentina serpentina* Linnaeus, 1758, but a comparison of this corresponding *18S rRNA* gene fragment shows only 96.3% identity between the two. The findings of this study compare with available morphological data of haemogregarine developmental stages and provide the first genetic data for this *Haemogregarina* sp. in this hemisphere.

Chapter III describes the retrieval and continuous cultivation of the erythrocytic stage of *B. bovis* and *B. bigemina* using the microaerophilous stationary phase (MASP) culture system after long-term cryostorage. *Babesia bovis* and *B. bigemina* are protozoa that are the causative agents of bovine babesiosis. *In vitro* cultivation of *Babesia* spp., premised on previous culture work with plasmodium, has had a great impact on the development of host-pathogen interaction research. This technique has led to a better understanding of the disease potential and the biology of the parasites.

In this study growth of *B. bovis* and *B. bigemina* recovered from cryopreservation and cultured in a humidified gas mixture of 2% oxygen, 5% carbon dioxide and 93% nitrogen atmosphere was monitored. The culture was maintained in erythrocytes and serum from a bovine donor. Cryopreserved *B. bovis* and *B. bigemina* stored nearly 30 years in liquid nitrogen at −196 C were successfully recovered in the MASP system. This study also confirmed previous observations that selection of a suitable bovine donor of erythrocytes and serum is critical to the success of the culture. Moreover, this was the first report of successfully introducing *Babesia* spp. resuscitated from frozen storage to culture in HL-1, a medium different from the one in which they were maintained prior to cryopreservation (M-199). The cryopreservation of cultured *Babesia* spp. stocks is well-recognized, but there is a paucity of information regarding how long these stocks will remain viable under extended storage.

The tick *Rhipicephalus (Boophilus) microplus*, which transmits *B. bovis* and *B. bigemina*, is currently enzootic in Puerto Rico. Despite ten decades of eradication attempts it remains an important cause of economical loss due to continued presence of the tick and its related tick vector pathogens. Moreover, fluctuation still exists between success and failure of control strategies.

Babesia spp. genotyping is a valid and needed resource as it permits strain/isolate discrimination and classification at the national or regional level. In Chapter IV, the lack of corresponding comparative *Babesia* data for the three genotyping markers (18S rDNA, rRNA ITS, and *msa-2b*) from key geographic sites limits the conclusions that may be drawn from this study at this time. This highlights the need for improved genome sequencing that will facilitate *Babesia* spp. population genetic studies.

In order to better understand the distribution of pathogenic *B. bovis* isolates, genotyping by sequencing multiple isolates from different geographic regions is recommended to understand the true genetic diversity of this pathogen in its geographic distribution. Furthermore, it would be highly advantageous to explore next generation sequencing to establish a sensitive genotyping system based on multi-locus typing.

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APPENDIX A

1. *Babesia* spp. GenBank® accession numbers for the 18S rRNA gene sequences.

Puerto Rico	Accession number	Puerto Rico	Accession number
PR1CL3	MH045741	PRh1086CL11	MH045772
PR1CL12	MH045742	PR11CL1BBIG	MH047814
PR2CL1	MH045743	PR12CL3BBIG	MH047815
PR2CL6	MH045744	PR25CL3BBIG	MH047816
PR2CL4	MH045745	PR28CL7BBIG	MH050387
PR4CL2	MH045746	PR36CL6BBIG	MH047817
PR4CL3	MH045747	PR37CL1BBIG	MH047818
PR5CL2	MH045748	PR38CL1BBIG	MH047819
PR5CL5	MH045749	Culture	Accession number
PR6CL3	MH045750	USDACL1AI	MH046902
PR6CL4	MH045751	USDACL8AI	MH046903
PR6CL10	MH045752	USDACL10AI	MH046904
PR7CL2	MH045753	HUSTCL10BBOV	MH046905
PR7CL3	MH045754	HUSTCL4BBOV	MH046906
PR7CL12	MH045755	1986CL1BBOV	MH046907
PR8CL1	MH045756	1986CL10BBOV	MH046908
PR8CL2	MH045757	1986CL11BBOV	MH046909
PR9CL2	MH045758	C123CL1BBOV	MH046910
PR17CL1	MH045759	C123CL3BBOV	MH046911
PR17CL2	MH045760	C275CL10BBOV	MH046912
PR19CL2	MH050386	C275CL12BBOV	MH046913
PR21CL2	MH045761	BBIGVIcl.4	MH050356
PR21CL3	MH045762		
PR24CL2	MH045763		
PR31CL2	MH045764		
PR32CL2	MH045765		
PR34CL1	MH045765		
PR34CL12	MH045767		
PR35CL1	MH045768		
PRhY500CL4	MH045769		
PRh890CL4	MH045770		
PRh1086CL4	MH045771		
Total	32		21

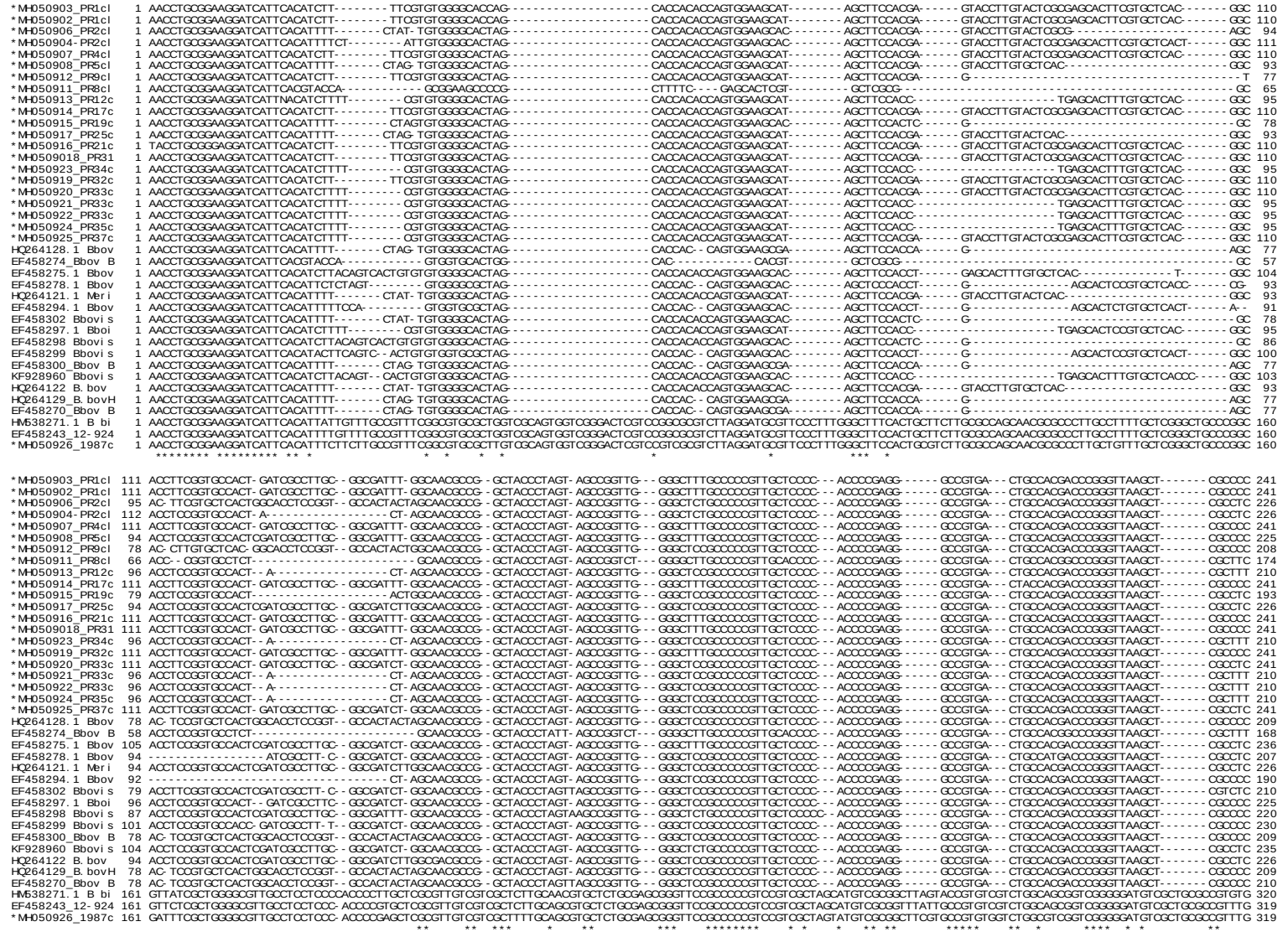
2. *Babesia* spp. GenBank® accession numbers for the ITS rRNA region

Isolate	Accession number
PR1CL12BBOV	MH050902
PR1CL5BBOV	MH050903
PR2CL3BBOV	MH050904
PR2CL10BBOV	MH050906
PR4CL3BBOV	MH050907
PR5CL1BBOV	MH050908
PR7CL3BBOV	MH0509010
PR8CL3BBOV	MH0509011
PR9CL3BBOV	MH0509012
PR12CL1BBOV	MH0509013
PR17CL1BBOV	MH0509014
PR19CL2BBOV	MH0509015
PR21CL1BBOV	MH0509016
PR25CL5BBOV	MH0509017
PR31CL10BBOV	MH0509018
PR33CL10BBOV	MH0509021
PR33CL12BBOV	MH0509022
PR34CL12BBOV	MH0509023
PR35CL2BBOV	MH0509024
PR37CL8BBOV	MH0509025
1987CL1BBIG	MH0509026
Total	21

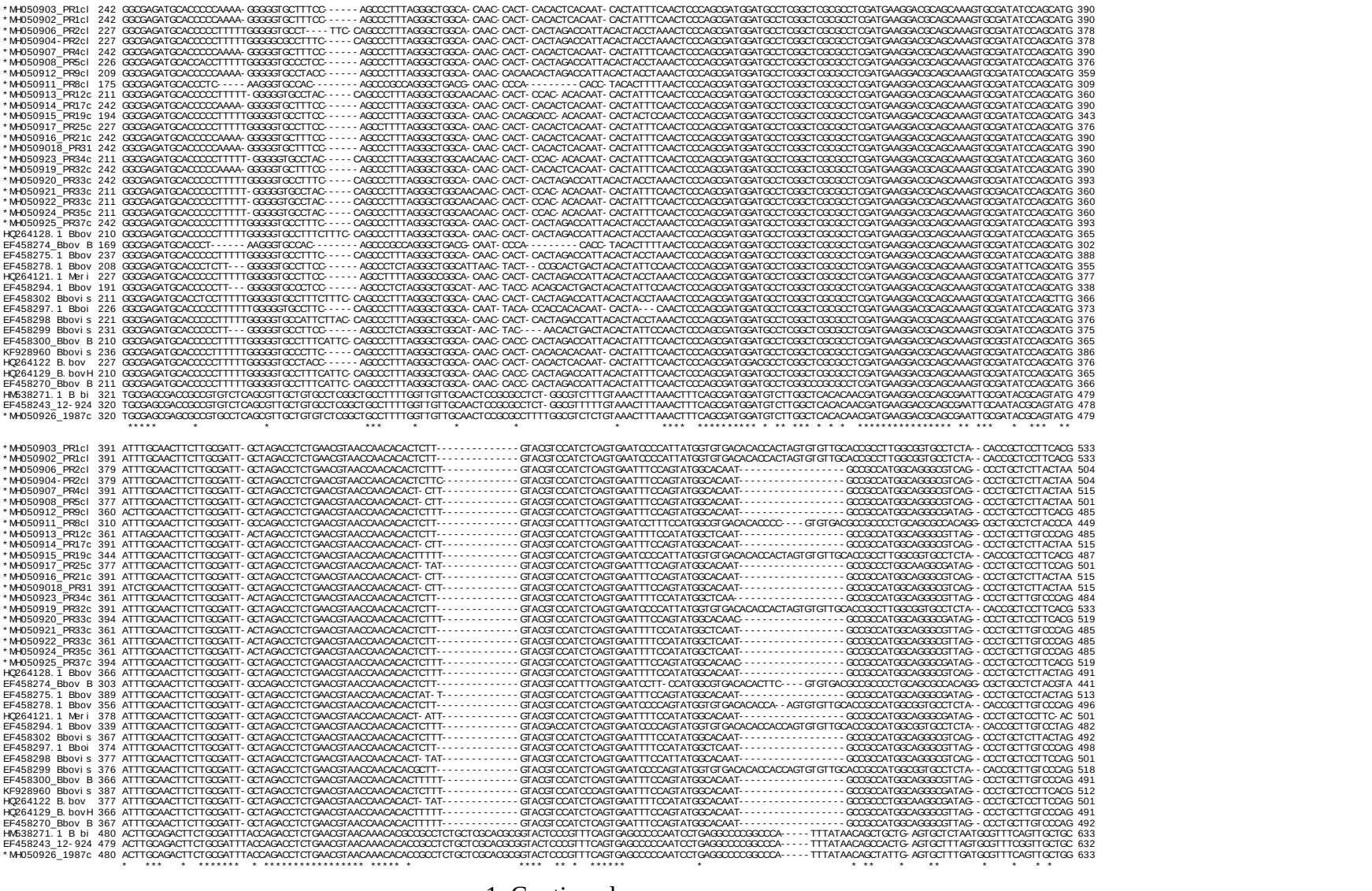
3. *Babesia bovis* GenBank® accession numbers for the *msa-2b* gene sequences.

Puerto Rico Isolate	**Accession no.**	**Culture isolates**	**Accession no.**
PR2 Clone 1	MH064400	USDA Ames Iowa (USDA) Clone	MH064403
PR2 Clone 2	MH064401	USDA Ames Iowa (USDA) Clone	MH064404
PR4 Clone 1	MH064402	USDA Ames Iowa (USDA) Clone	MH064405
PR4 Clone 4	MH064414	Mexico BbovC275 Clone 1	MH064407
PR5 Clone 1	MH064415	Mexico BbovC275 clone 11	MH064408
PR6 Clone 1	MH064416	Mexico Bbov1986 Clone 1	MH064409
PR6 Clone 2	MH064417	Mexico HUASTECA Clone 2	MH064410
PR8 Clone 5	MH064418	Mexico HUASTECA Clone 3	MH064411
PR9 Clone 2	MH064419	Mexico BbovC123 Clone 1	MH064412
PR9 Clone 3	MH064420	Mexico BbovC123Clone 2	MH064413
PR9 Clone 5	MH064421		
PR19 Clone 2	MH064422		
PR22 Clone 1	MH064423		
PR22 Clone 2	MH064424		
PR23 Clone 1	MH064425		
PR24 Clone 1	MH064426		
PR24 Clone 4	MH064427		
PR31 Clone 1	MH064428		
PR32 Clone 9	MH064429		
PRh1086 Clone 2	MH064430		
Total	20		10

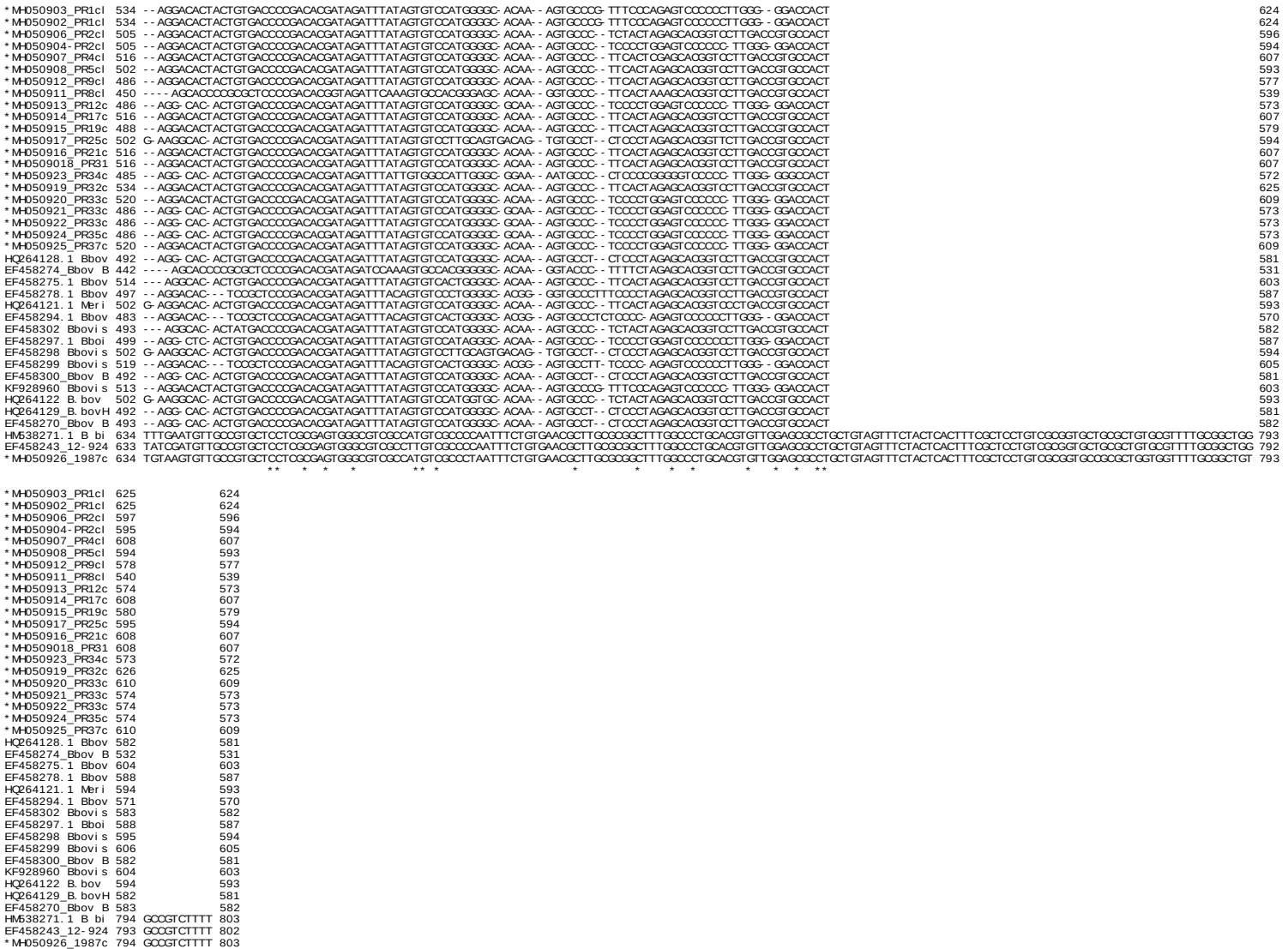
APPENDIX B



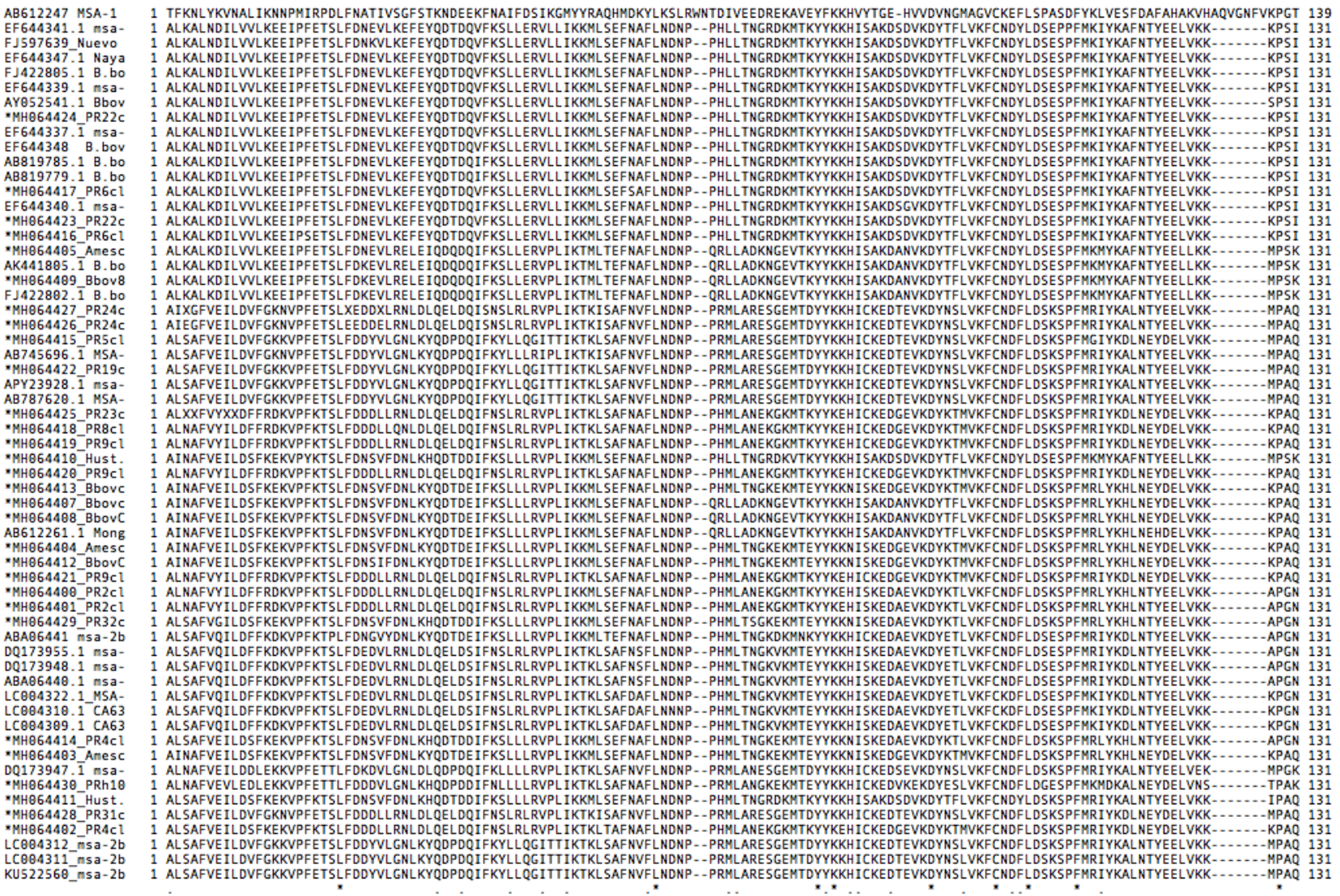
1. ClustalW multiple sequence alignment of *Babesia* spp. ITS1-ITS2 rRNA region



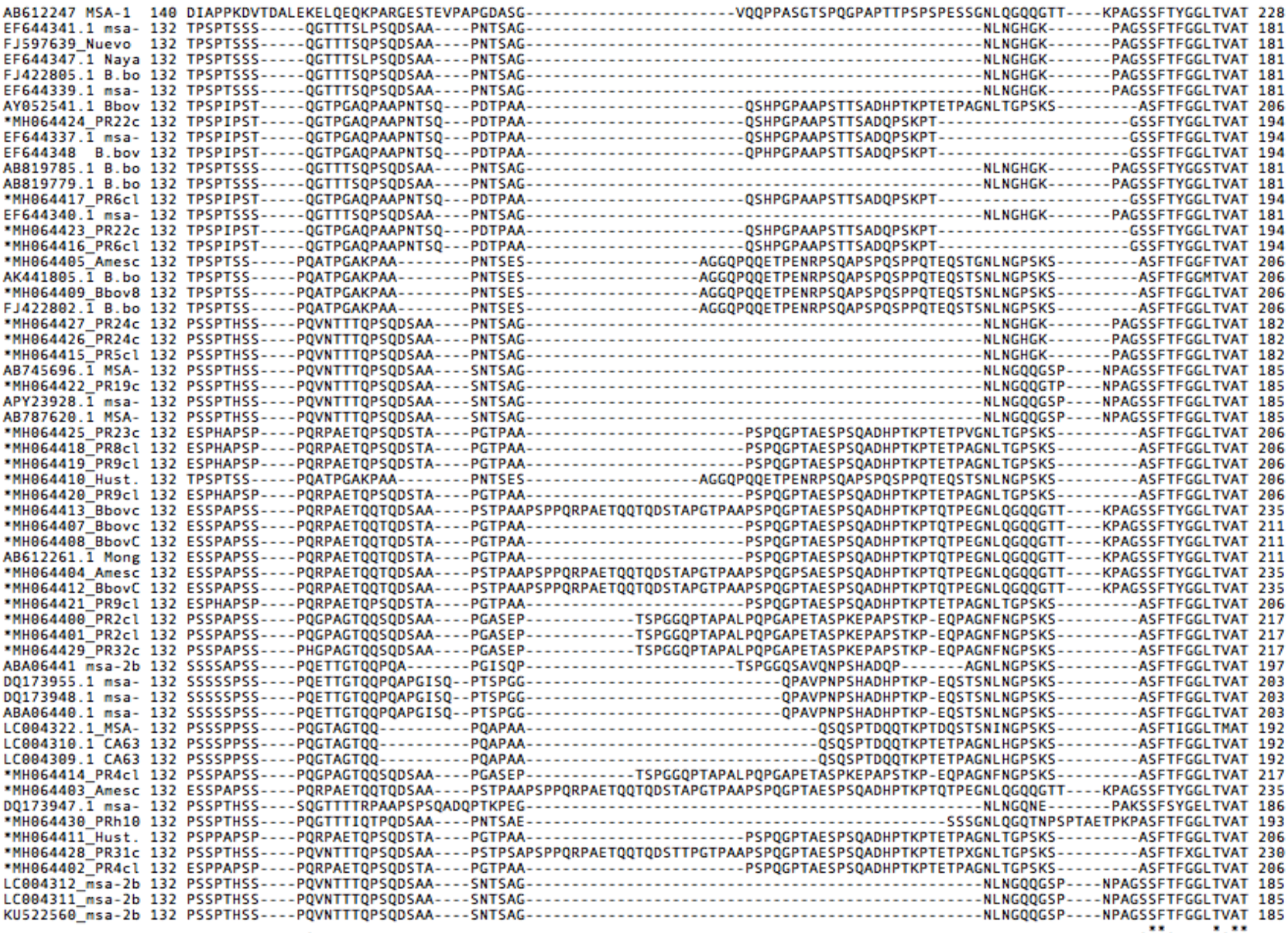
1. Continued



1. Continued

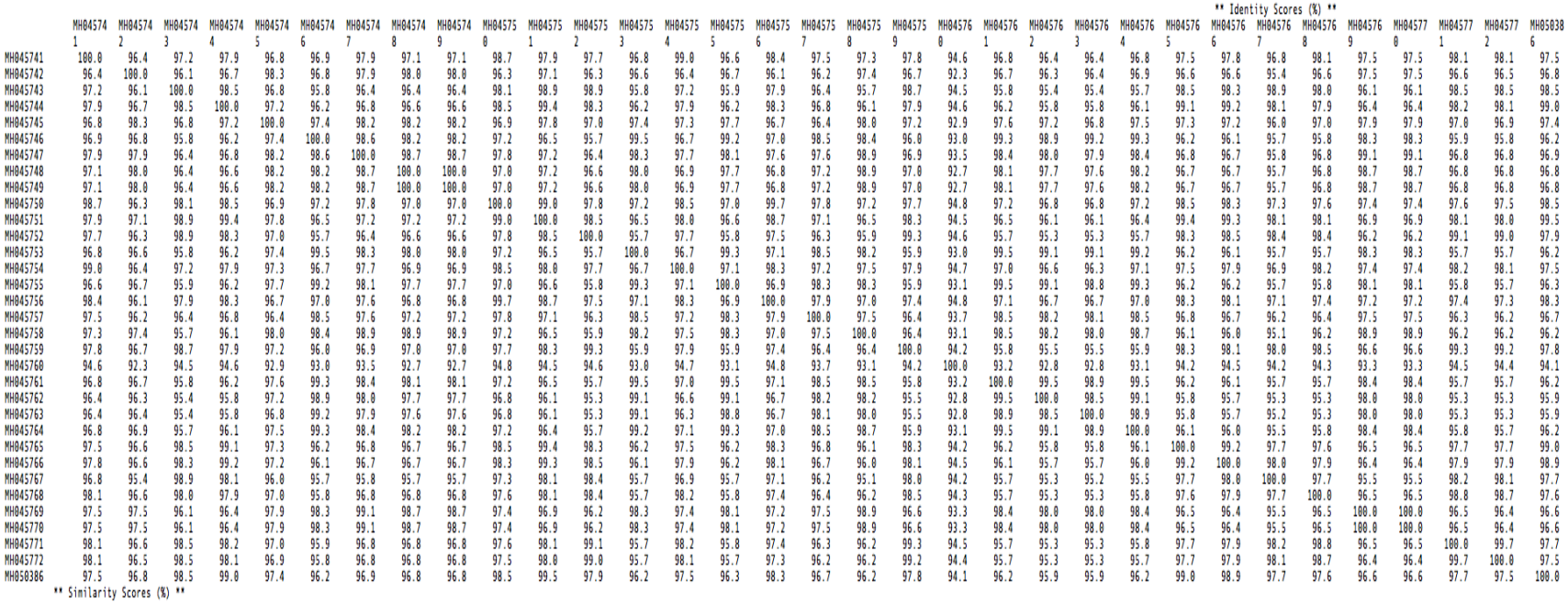


2. MUSCLE multiple alignment of deduced amino acid sequences for *Babesia* spp. MSA-2b.



2. Continued

APPENDIX C



** Identity Scores (%) **

	MH04574 1	MH04574 2	MH04574 3	MH04574 4	MH04574 5	MH04574 6	MH04574 7	MH04574 8	MH04574 9	MH04575 0	MH04575 1	MH04575 2	MH04575 3	MH04575 4	MH04575 5	MH04575 6	MH04575 7	MH04575 8	MH04575 9	MH04576 0	MH04576 1	MH04576 2	MH04576 3	MH04576 4	MH04576 5	MH04576 6	MH04576 7	MH04576 8	MH04576 9	MH04577 0	MH04577 1	MH04577 2	MH05038 6
MH045741	100.0	96.4	97.2	97.9	96.8	96.9	97.9	97.1	97.1	98.7	97.9	97.7	96.8	99.0	96.6	98.4	97.5	97.3	97.8	94.6	96.8	96.4	96.4	96.8	97.5	97.8	96.8	98.1	97.5	97.5	98.1	98.1	97.5
MH045742	96.4	100.0	96.1	96.7	98.3	96.8	97.9	98.0	98.0	96.3	97.1	96.3	96.6	96.4	96.7	96.1	96.2	97.4	96.7	92.3	96.7	96.3	96.4	96.9	96.6	96.6	95.4	96.6	97.5	97.5	96.6	96.5	96.8
MH045743	97.2	96.1	100.0	98.5	96.8	95.8	96.4	96.4	96.4	98.1	98.9	98.9	95.8	97.2	95.9	97.9	96.4	95.7	98.7	94.5	95.8	95.4	95.4	95.7	98.5	98.3	98.9	98.0	96.1	96.1	98.5	98.5	98.5
MH045744	97.9	96.7	98.5	100.0	97.2	96.2	96.8	96.6	96.6	98.5	99.4	98.3	96.2	97.9	96.2	98.3	96.8	96.1	97.9	94.6	96.2	95.8	95.8	96.1	99.1	99.2	98.1	97.9	96.4	96.4	98.2	98.1	99.0
MH045745	96.8	98.3	96.8	97.2	100.0	97.4	98.2	98.2	98.2	96.9	97.8	97.0	97.4	97.3	97.7	96.7	96.4	98.0	97.2	92.9	97.6	97.2	96.8	97.5	97.3	97.2	96.0	97.0	97.9	97.9	97.0	96.9	97.4
MH045746	96.9	96.8	95.8	96.2	97.4	100.0	98.6	98.2	98.2	97.2	96.5	95.7	99.5	96.7	99.2	97.0	98.5	98.4	96.0	93.0	99.3	98.9	99.2	99.3	96.2	96.1	95.7	95.8	98.3	98.3	95.9	95.8	96.2
MH045747	97.9	97.9	96.4	96.8	98.2	98.6	100.0	98.7	98.7	97.8	97.2	96.4	98.3	97.7	98.1	97.6	97.6	98.9	96.9	93.5	98.4	98.0	97.9	98.4	96.8	96.7	95.8	96.8	99.1	99.1	96.8	96.8	96.9
MH045748	97.1	98.0	96.4	96.6	98.2	98.2	98.7	100.0	100.0	97.0	97.2	96.6	98.0	96.9	97.7	96.8	97.2	98.9	97.0	92.7	98.1	97.7	97.6	98.2	96.7	96.7	95.7	96.8	98.7	98.7	96.8	96.8	96.8
MH045749	97.1	98.0	96.4	96.6	98.2	98.2	98.7	100.0	100.0	97.0	97.2	96.6	98.0	96.9	97.7	96.8	97.2	98.9	97.0	92.7	98.1	97.7	97.6	98.2	96.7	96.7	95.7	96.8	98.7	98.7	96.8	96.8	96.8
MH045750	98.7	96.3	98.1	98.5	96.9	97.2	97.8	97.0	97.0	100.0	99.0	97.8	97.2	98.5	97.0	99.7	97.8	97.2	97.7	94.8	97.2	96.8	96.8	97.2	98.5	98.3	97.3	97.6	97.4	97.4	97.6	97.5	98.5
MH045751	97.9	97.1	98.9	99.4	97.8	96.5	97.2	97.2	97.2	99.0	100.0	98.5	96.5	98.0	96.6	98.7	97.1	96.5	98.3	94.5	96.5	96.1	96.1	96.4	99.4	99.3	98.1	98.1	96.9	96.9	98.1	98.0	99.5
MH045752	97.7	96.3	98.9	98.3	97.0	95.7	96.4	96.6	96.6	97.8	98.5	100.0	95.7	97.7	95.8	97.5	96.3	95.9	99.3	94.6	95.7	95.3	95.3	95.7	98.3	98.5	98.4	98.4	96.2	96.2	99.1	99.0	97.9
MH045753	96.8	96.6	95.8	96.2	97.4	99.5	98.3	98.0	98.0	97.2	96.5	95.7	100.0	96.7	99.3	97.1	98.5	98.2	95.9	93.0	99.5	99.1	99.1	99.2	96.2	96.1	95.7	95.7	98.3	98.3	95.7	95.7	96.2
MH045754	99.0	96.4	97.2	97.9	97.3	96.7	97.7	96.9	96.9	98.5	98.0	97.7	96.7	100.0	97.1	98.3	97.2	97.5	97.9	94.7	97.0	96.6	96.3	97.1	97.5	97.9	96.9	98.2	97.4	97.4	98.2	98.1	97.5
MH045755	96.6	96.7	95.9	96.2	97.7	99.2	98.1	97.7	97.7	97.0	96.6	95.8	99.3	97.1	100.0	96.9	98.3	98.3	95.9	93.1	99.5	99.1	98.8	99.3	96.2	96.2	95.7	95.8	98.1	98.1	95.8	95.7	96.3
MH045756	98.4	96.1	97.9	98.3	96.7	97.0	97.6	96.8	96.8	99.7	98.7	97.5	97.1	98.3	96.9	100.0	97.9	97.0	97.4	94.8	97.1	96.7	96.7	97.0	98.3	98.1	97.1	97.4	97.2	97.2	97.4	97.3	98.3
MH045757	97.5	96.2	96.4	96.8	96.4	98.5	97.6	97.2	97.2	97.8	97.1	96.3	98.5	97.2	98.3	97.9	100.0	97.5	96.4	93.7	98.5	98.2	98.1	98.5	96.8	96.7	96.2	96.4	97.5	97.5	96.3	96.2	96.7
MH045758	97.3	97.4	95.7	96.1	98.0	98.4	98.9	98.9	98.9	97.2	96.5	95.9	98.2	97.5	98.3	97.0	97.5	100.0	96.4	93.1	98.5	98.2	98.0	98.7	96.1	96.0	95.1	96.2	98.9	98.9	96.2	96.2	96.2
MH045759	97.8	96.7	98.7	97.9	97.2	96.0	96.9	97.0	97.0	97.7	98.3	99.3	95.9	97.9	95.9	97.4	96.4	96.4	100.0	94.2	95.8	95.5	95.5	95.9	98.3	98.1	98.0	98.5	96.6	96.6	99.3	99.2	97.8
MH045760	94.6	92.3	94.5	94.6	92.9	93.0	93.5	92.7	92.7	94.8	94.5	94.6	93.0	94.7	93.1	94.8	93.7	93.1	94.2	100.0	93.2	92.8	92.8	93.1	94.2	94.5	94.2	94.3	93.3	93.3	94.5	94.4	94.1
MH045761	96.8	96.7	95.8	96.2	97.6	99.3	98.4	98.1	98.1	97.2	96.5	95.7	99.5	97.0	99.5	97.1	98.5	98.5	95.8	93.2	100.0	99.5	98.9	99.5	96.2	96.1	95.7	95.7	98.4	98.4	95.7	95.7	96.2
MH045762	96.4	96.3	95.4	95.8	97.2	98.9	98.0	97.7	97.7	96.8	96.1	95.3	99.1	96.6	99.1	96.7	98.2	98.2	95.5	92.8	99.5	100.0	98.5	99.1	95.8	95.7	95.3	95.3	98.0	98.0	95.3	95.3	95.9
MH045763	96.4	96.4	95.4	95.8	96.8	99.2	97.9	97.6	97.6	96.8	96.1	95.3	99.1	96.3	98.8	96.7	98.1	98.0	95.5	92.8	98.9	98.5	100.0	98.9	95.8	95.7	95.2	95.3	98.0	98.0	95.3	95.3	95.9
MH045764	96.8	96.9	95.7	96.1	97.5	99.3	98.4	98.2	98.2	97.2	96.4	95.7	99.2	97.1	99.3	97.0	98.5	98.7	95.9	93.1	99.5	99.1	98.9	100.0	96.1	96.0	95.5	95.8	98.4	98.4	95.8	95.7	96.2
MH045765	97.5	96.6	98.5	99.1	97.3	96.2	96.8	96.7	96.7	98.5	99.4	98.3	96.2	97.5	96.2	98.3	96.8	96.1	98.3	94.2	96.2	95.8	95.8	96.1	100.0	99.2	97.7	97.6	96.5	96.5	96.5	97.7	99.0
MH045766	97.8	96.6	98.3	99.2	97.2	96.1	96.7	96.7	96.7	98.3	99.3	98.5	96.1	97.9	96.2	98.1	96.7	96.0	98.1	94.5	96.1	95.7	95.7	96.0	99.2	100.0	98.0	97.9	96.4	96.4	97.9	97.9	98.9
MH045767	96.8	95.4	98.9	98.1	96.0	95.7	95.8	95.7	95.7	97.3	98.1	98.4	95.7	96.9	95.7	97.1	96.2	95.1	98.0	94.2	95.7	95.3	95.2	95.5	97.7	98.0	100.0	97.7	95.5	95.5	98.2	98.1	97.7
MH045768	98.1	96.6	98.0	97.9	97.0	95.8	96.8	96.8	96.8	97.6	98.1	98.4	95.7	98.2	95.8	97.4	96.4	96.2	98.5	94.3	95.7	95.3	95.3	95.8	97.6	97.9	97.7	100.0	96.5	96.5	98.8	98.7	97.6
MH045769	97.5	97.5	96.1	96.4	97.9	98.3	99.1	98.7	98.7	97.4	96.9	96.2	98.3	97.4	98.1	97.2	97.5	98.9	96.6	93.3	98.4	98.0	98.0	98.4	96.5	96.4	95.5	96.5	100.0	100.0	96.5	96.4	96.6
MH045770	97.5	97.5	96.1	96.4	97.9	98.3	99.1	98.7	98.7	97.4	96.9	96.2	98.3	97.4	98.1	97.2	97.5	98.9	96.6	93.3	98.4	98.0	98.0	98.4	96.5	96.4	95.5	96.5	100.0	100.0	96.5	96.4	96.6
MH045771	98.1	96.6	98.5	98.2	97.0	95.9	96.8	96.8	96.8	97.6	98.1	99.1	95.7	98.2	95.8	97.4	96.3	96.2	99.3	94.5	95.7	95.3	95.3	95.8	97.7	97.9	98.2	98.8	96.5	96.5	100.0	99.7	97.7
MH045772	98.1	96.5	98.5	98.1	96.9	95.8	96.8	96.8	96.8	97.5	98.0	99.0	95.7	98.1	95.7	97.3	96.2	96.2	99.2	94.4	95.7	95.3	95.3	95.7	97.7	97.9	98.1	98.7	96.4	96.4	99.7	100.0	97.5
MH050386	97.5	96.8	98.5	99.0	97.4	96.2	96.9	96.8	96.8	98.5	99.5	97.9	96.2	97.5	96.3	98.3	96.7	96.2	97.8	94.1	96.2	95.9	95.9	96.2	99.0	98.9	97.7	97.6	96.6	96.6	97.7	97.5	100.0

** Similarity Scores (%) **

1. *B. bovis 18S rRNA* gene pairwise identity scores among the Puerto Rico isolates.

								** Identity Scores (%) **				
	>MH0469 02	>MH0469 03	>MH0469 04	>MH0469 05	>MH0469 06	>MH0469 07	>MH0469 08	>MH0469 09	>MH0469 10	>MH0469 11	>MH0469 13	>MH0469 12
>MH046902	100.0	99.4	99.5	98.2	97.9	98.7	98.1	98.5	99.7	99.9	98.4	98.7
>MH046903	99.4	100.0	99.2	98.1	97.9	98.7	98.0	98.5	99.5	99.4	98.5	98.6
>MH046904	99.5	99.2	100.0	97.9	97.7	98.5	97.9	98.3	99.5	99.5	98.2	98.5
>MH046905	98.2	98.1	97.9	100.0	98.7	99.1	98.5	99.1	98.1	98.2	99.0	99.0
>MH046906	97.9	97.9	97.7	98.7	100.0	98.8	98.3	99.2	97.9	97.9	98.7	98.7
>MH046907	98.7	98.7	98.5	99.1	98.8	100.0	99.3	99.6	98.7	98.7	99.5	99.8
>MH046908	98.1	98.0	97.9	98.5	98.3	99.3	100.0	99.1	98.0	98.1	99.0	99.1
>MH046909	98.5	98.5	98.3	99.1	99.2	99.6	99.1	100.0	98.5	98.5	99.5	99.5
>MH046910	99.7	99.5	99.5	98.1	97.9	98.7	98.0	98.5	100.0	99.8	98.5	98.6
>MH046911	99.9	99.4	99.5	98.2	97.9	98.7	98.1	98.5	99.8	100.0	98.4	98.7
>MH046913	98.4	98.5	98.2	99.0	98.7	99.5	99.0	99.5	98.5	98.4	100.0	99.5
>MH046912	98.7	98.6	98.5	99.0	98.7	99.8	99.1	99.5	98.6	98.7	99.5	100.0

** Similarity Scores (%) **

2. Pairwise identity scores for *B. bovis 18Sr RNA* gene among the archived samples.

** Identity Scores (%) **

	MH050902	MH050903	MH050904	MH050906	MH050907	MH050908	MH050911	MH050912	MH050913	MH050914	MH050915	MH050916	MH050917	MH050918	MH050919	MH050920	MH050921	MH050922	MH050923	MH050924	MH050925
MH050902	100.0	100.0	84.9	83.2	90.7	83.8	71.3	82.2	82.7	90.6	84.9	90.6	82.7	90.7	97.3	89.5	82.9	83.1	81.8	83.1	89.5
MH050903	100.0	100.0	84.9	83.2	90.7	83.8	71.3	82.2	82.7	90.6	84.9	90.6	82.7	90.7	97.3	89.5	82.9	83.1	81.8	83.1	89.5
MH050904	84.9	84.9	100.0	96.8	89.6	89.4	71.0	89.4	90.3	89.2	82.1	89.2	83.8	89.4	83.5	94.1	90.4	90.6	88.4	90.6	94.1
MH050906	83.2	83.2	96.8	100.0	91.5	91.4	73.1	92.1	87.6	91.4	84.7	91.4	85.6	91.5	85.6	91.7	87.8	88.0	86.5	88.0	91.7
MH050907	90.7	90.7	89.6	91.5	100.0	92.5	72.2	89.2	86.2	99.5	81.1	99.5	89.7	99.7	93.4	92.3	86.4	86.5	85.4	86.5	92.3
MH050908	83.8	83.8	89.4	91.4	92.5	100.0	73.0	87.3	87.1	92.3	84.2	92.3	92.5	92.5	86.5	90.8	87.2	87.4	86.2	87.4	90.8
MH050911	71.3	71.3	71.0	73.1	72.2	73.0	100.0	70.9	72.2	72.1	74.5	72.1	71.0	72.2	73.8	69.3	72.4	72.6	71.7	72.6	69.3
MH050912	82.2	82.2	89.4	92.1	89.2	87.3	70.9	100.0	84.9	89.0	86.3	89.0	83.4	89.2	84.9	89.4	85.1	85.2	84.1	85.2	89.4
MH050913	82.7	82.7	90.3	87.6	86.2	87.1	72.2	84.9	100.0	85.9	82.6	85.9	86.8	86.0	81.3	88.5	99.5	99.7	97.4	99.7	88.5
MH050914	90.6	90.6	89.2	91.4	99.5	92.3	72.1	89.0	85.9	100.0	80.9	99.3	89.5	99.5	93.3	92.0	86.0	86.2	85.1	86.2	92.0
MH050915	84.9	84.9	82.1	84.7	81.1	84.2	74.5	86.3	82.6	80.9	100.0	80.9	82.9	81.1	87.6	80.7	82.8	83.0	82.0	83.0	80.7
MH050916	90.6	90.6	89.2	91.4	99.5	92.3	72.1	89.0	85.9	99.3	80.9	100.0	89.5	99.5	93.3	92.0	86.0	86.2	85.1	86.2	92.0
MH050917	82.7	82.7	83.8	85.6	89.7	92.5	71.0	83.4	86.8	89.5	82.9	89.5	100.0	89.7	85.1	87.3	86.9	87.1	86.3	87.1	87.3
MH050918	90.7	90.7	89.4	91.5	99.7	92.5	72.2	89.2	86.0	99.5	81.1	99.5	89.7	100.0	93.4	92.2	86.2	86.4	85.2	86.4	92.2
MH050919	97.3	97.3	83.5	85.6	93.4	86.5	73.8	84.9	81.3	93.3	87.6	93.3	85.1	93.4	100.0	88.1	81.5	81.7	80.7	81.7	88.1
MH050920	89.5	89.5	94.1	91.7	92.3	90.8	69.3	89.4	88.5	92.0	80.7	92.0	87.3	92.2	88.1	100.0	88.7	88.9	86.9	88.9	100.0
MH050921	82.9	82.9	90.4	87.8	86.4	87.2	72.4	85.1	99.5	86.0	82.8	86.0	86.9	86.2	81.5	88.7	100.0	99.8	97.6	99.8	88.7
MH050922	83.1	83.1	90.6	88.0	86.5	87.4	72.6	85.2	99.7	86.2	83.0	86.2	87.1	86.4	81.7	88.9	99.8	100.0	97.7	100.0	88.9
MH050923	81.8	81.8	88.4	86.5	85.4	86.2	71.7	84.1	97.4	85.1	82.0	85.1	86.3	85.2	80.7	86.9	97.6	97.7	100.0	97.7	86.9
MH050924	83.1	83.1	90.6	88.0	86.5	87.4	72.6	85.2	99.7	86.2	83.0	86.2	87.1	86.4	81.7	88.9	99.8	100.0	97.7	100.0	88.9
MH050925	89.5	89.5	94.1	91.7	92.3	90.8	69.3	89.4	88.5	92.0	80.7	92.0	87.3	92.2	88.1	100.0	88.7	88.9	86.9	88.9	100.0

** Similarity Scores (%) **

4. Pairwise identity scores for *B. bovis* ITS region among the Puerto Rico isolates.

** Identity Scores (%) **

	*MH050903	*MH050902	*MH050906	*MH050904	*MH050907	*MH050908	*MH050912	*MH050911	*MH050913	*MH050914	*MH050915	*MH050917
*MH050903	100.0	100.0	79.6	84.0	90.1	83.5	79.3	68.3	80.0	90.0	84.3	82.3
*MH050902	100.0	100.0	79.6	84.0	90.1	83.5	79.3	68.3	80.0	90.0	84.3	82.3
*MH050906	79.6	79.6	100.0	87.8	88.2	93.0	91.0	69.2	79.9	88.0	82.2	88.2
*MH050904	84.0	84.0	87.8	100.0	88.8	88.0	82.3	68.1	89.6	88.5	81.8	83.5
*MH050907	90.1	90.1	88.2	88.8	100.0	92.6	86.8	68.9	83.4	99.5	80.0	89.7
*MH050908	83.5	83.5	93.0	88.0	92.6	100.0	89.1	71.1	81.2	92.5	83.3	92.6
*MH050912	79.3	79.3	91.0	82.3	86.8	89.1	100.0	70.8	80.8	86.6	84.9	86.0
*MH050911	68.3	68.3	69.2	68.1	68.9	71.1	70.8	100.0	68.6	68.8	75.9	68.6
*MH050913	80.0	80.0	79.9	89.6	83.4	81.2	80.8	68.6	100.0	83.1	81.5	79.9
*MH050914	90.0	90.0	88.0	88.5	99.5	92.5	86.6	68.8	83.1	100.0	79.9	89.6
*MH050915	84.3	84.3	82.2	81.8	80.0	83.3	84.9	75.9	81.5	79.9	100.0	81.4
*MH050917	82.3	82.3	88.2	83.5	89.7	92.6	86.0	68.6	79.9	89.6	81.4	100.0
*MH050916	90.0	90.0	88.0	88.5	99.5	92.5	86.6	68.8	83.1	99.3	79.9	89.6
*MH0509018	90.1	90.1	88.2	88.6	99.7	92.6	86.8	68.9	83.2	99.5	80.0	89.7
*MH050923	79.0	79.0	78.7	87.8	82.6	80.4	80.0	68.1	97.4	82.2	80.9	79.4
*MH050919	97.3	97.3	81.9	83.2	92.8	86.2	82.0	70.7	79.2	92.7	87.0	84.7
*MH050920	88.7	88.7	87.6	94.1	91.5	89.8	86.0	66.9	88.2	91.2	80.5	87.4
*MH050921	80.2	80.2	80.0	89.8	83.6	81.4	81.0	68.8	99.5	83.2	81.7	80.1
*MH050922	80.3	80.3	80.2	89.9	83.7	81.5	81.1	69.0	99.7	83.4	81.9	80.3
*MH050924	80.3	80.3	80.2	89.9	83.7	81.5	81.1	69.0	99.7	83.4	81.9	80.3
*MH050925	88.7	88.7	87.6	94.1	91.5	89.8	86.0	66.9	88.2	91.2	80.5	87.4
HQ264128_TX	76.1	76.1	93.5	83.6	84.0	89.8	92.0	70.3	82.2	83.8	83.2	86.7
EF458275_Arg.	80.3	80.3	89.0	85.2	88.2	93.1	87.0	69.8	81.0	88.0	81.6	89.8
EF458278_Aust.	80.9	80.9	74.2	75.6	78.2	76.1	75.6	69.6	76.1	78.1	81.8	75.5
EF458294_Aust.	80.7	80.7	71.8	78.4	73.9	72.2	73.5	68.5	79.0	73.7	81.8	70.6
HQ264121_Mex.	82.0	82.0	91.7	86.4	89.9	96.0	89.1	70.2	81.7	89.7	83.2	94.1
EF458297_Mex	81.4	81.4	81.0	87.3	85.0	83.1	82.9	66.9	93.4	84.7	80.3	81.2
EF458302_Mex	78.1	78.1	90.5	84.4	86.1	91.8	88.6	70.5	82.9	85.9	84.0	88.2
EF458298_Urug.	78.2	78.2	86.8	82.1	84.8	90.0	87.3	69.7	80.7	84.7	82.8	90.3
EF458299_S Afr.	85.5	85.5	73.4	78.6	78.8	76.3	75.3	68.5	79.3	78.6	81.7	75.6
EF458300_TX	78.7	78.7	91.2	85.0	86.7	92.3	89.3	71.0	83.5	86.5	84.7	88.9
HQ264122_Mex.	82.6	82.6	89.7	84.0	89.9	92.9	86.6	69.2	81.2	89.7	82.2	96.1
KF928960_India	84.2	84.2	82.6	87.4	86.6	85.4	83.8	67.0	88.9	86.5	80.7	83.8
HQ264129_TX	76.4	76.4	92.3	82.5	84.0	88.8	91.8	70.3	82.7	83.8	83.6	87.7
EF458274_Turk	68.0	68.0	69.4	68.2	68.8	71.1	70.5	93.3	69.0	68.6	75.6	68.3
EF458270_Mex	76.1	76.1	92.0	82.2	83.7	88.5	91.5	70.0	82.4	83.5	83.3	87.4

	*MH050916	*MH0509018	*MH050923	*MH050919	*MH050920	*MH050921	*MH050922	*MH050924	*MH050925	HQ264128_TX	EF458275_Arg.	EF458278_Aust.
*MH050903	90.0	90.1	79.0	97.3	88.7	80.2	80.3	80.3	88.7	76.1	80.3	80.9
*MH050902	90.0	90.1	79.0	97.3	88.7	80.2	80.3	80.3	88.7	76.1	80.3	80.9
*MH050906	88.0	88.2	78.7	81.9	87.6	80.0	80.2	80.2	87.6	93.5	89.0	74.2
*MH050904	88.5	88.6	87.8	83.2	94.1	89.8	89.9	89.9	94.1	83.6	85.2	75.6
*MH050907	99.5	99.7	82.6	92.8	91.5	83.6	83.7	83.7	91.5	84.0	88.2	78.2
*MH050908	92.5	92.6	80.4	86.2	89.8	81.4	81.5	81.5	89.8	89.8	93.1	76.1
*MH050912	86.6	86.8	80.0	82.0	86.0	81.0	81.1	81.1	86.0	92.0	87.0	75.6
*MH050911	68.8	68.9	68.1	70.7	66.9	68.8	69.0	69.0	66.9	70.3	69.8	69.6
*MH050913	83.1	83.2	97.4	79.2	88.2	99.5	99.7	99.7	88.2	82.2	81.0	76.1
*MH050914	99.3	99.5	82.2	92.7	91.2	83.2	83.4	83.4	91.2	83.8	88.0	78.1
*MH050915	79.9	80.0	80.9	87.0	80.5	81.7	81.9	81.9	80.5	83.2	81.6	81.8
*MH050917	89.6	89.7	79.4	84.7	87.4	80.1	80.3	80.3	87.4	86.7	89.8	75.5
*MH050916	100.0	99.5	82.2	92.7	91.2	83.2	83.4	83.4	91.2	83.8	88.0	78.1
*MH0509018	99.5	100.0	82.4	92.8	91.4	83.4	83.6	83.6	91.4	84.0	88.2	78.2
*MH050923	82.2	82.4	100.0	78.6	86.6	97.6	97.7	97.7	86.6	81.7	80.2	75.1
*MH050919	92.7	92.8	78.6	100.0	87.9	79.4	79.5	79.5	87.9	78.4	82.9	83.1
*MH050920	91.2	91.4	86.6	87.9	100.0	88.4	88.5	88.5	100.0	83.9	88.4	77.6
*MH050921	83.2	83.4	97.6	79.4	88.4	100.0	99.8	99.8	88.4	82.4	81.2	76.2
*MH050922	83.4	83.6	97.7	79.5	88.5	99.8	100.0	100.0	88.5	82.5	81.3	76.4
*MH050924	83.4	83.6	97.7	79.5	88.5	99.8	100.0	100.0	88.5	82.5	81.3	76.4
*MH050925	91.2	91.4	86.6	87.9	100.0	88.4	88.5	88.5	100.0	83.9	88.4	77.6
HQ264128_TX	83.8	84.0	81.7	78.4	83.9	82.4	82.5	82.5	83.9	100.0	87.4	75.8
EF458275_Arg.	88.0	88.2	80.2	82.9	88.4	81.2	81.3	81.3	88.4	87.4	100.0	76.2
EF458278_Aust.	78.1	78.2	75.1	83.1	77.6	76.2	76.4	76.4	77.6	75.8	76.2	100.0
EF458294_Aust.	73.7	73.9	77.5	79.0	76.1	79.2	79.3	79.3	76.1	73.6	71.7	90.2
HQ264121_Mex.	89.7	89.9	80.9	84.7	90.1	81.9	82.0	82.0	90.1	89.5	92.6	75.6
EF458297_Mex	84.7	84.8	91.7	80.8	89.7	93.6	93.7	93.7	89.7	83.8	82.7	78.0
EF458302_Mex	85.9	86.1	81.7	80.4	86.5	83.0	83.2	83.2	86.5	92.5	91.0	77.3
EF458298_Urug.	84.7	84.8	80.2	80.5	85.1	80.8	81.0	81.0	85.1	89.8	91.8	76.4
EF458299_S Afr.	78.6	78.8	77.8	83.8	81.0	79.4	79.6	79.6	81.0	75.6	77.5	90.2
EF458300_TX	86.5	86.7	82.3	81.0	87.1	83.7	83.8	83.8	87.1	93.2	91.6	77.7
HQ264122_Mex.	89.7	89.9	80.1	85.0	87.5	81.4	81.5	81.5	87.5	87.0	90.6	75.5
KF928960_India	86.5	86.6	87.7	82.5	91.5	89.1	89.2	89.2	91.5	82.9	87.0	78.6
HQ264129_TX	83.8	84.0	82.2	78.7	83.6	82.9	83.0	83.0	83.6	97.6	86.9	76.9
EF458274_Turk	68.6	68.8	68.5	70.2	66.8	69.1	69.3	69.3	66.8	70.5	69.8	68.7
EF458270_Mex	83.5	83.7	81.9	78.4	83.3	82.6	82.7	82.7	83.3	97.3	86.6	76.6

	EF458294_Aust.	HQ264121_Mex.	EF458297_Mex	EF458302_Mex	EF458298_Urug.	EF458299_S Afr	EF458300_TX	HQ264122_Mex.	KF928960_India	HQ264129_TX	EF458274_Turk	EF458270_Mex
*MH050903	80.7	82.0	81.4	78.1	78.2	85.5	78.7	82.6	84.2	76.4	68.0	76.1
*MH050902	80.7	82.0	81.4	78.1	78.2	85.5	78.7	82.6	84.2	76.4	68.0	76.1
*MH050906	71.8	91.7	81.0	90.5	86.8	73.4	91.2	89.7	82.6	92.3	69.4	92.0
*MH050904	78.4	86.4	87.3	84.4	82.1	78.6	85.0	84.0	87.4	82.5	68.2	82.2
*MH050907	73.9	89.9	85.0	86.1	84.8	78.8	86.7	89.9	86.6	84.0	68.8	83.7
*MH050908	72.2	96.0	83.1	91.8	90.0	76.3	92.3	92.9	85.4	88.8	71.1	88.5
*MH050912	73.5	89.1	82.9	88.6	87.3	75.3	89.3	86.6	83.8	91.8	70.5	91.5
*MH050911	68.5	70.2	66.9	70.5	69.7	68.5	71.0	69.2	67.0	70.3	93.3	70.0
*MH050913	79.0	81.7	93.4	82.9	80.7	79.3	83.5	81.2	88.9	82.7	69.0	82.4
*MH050914	73.7	89.7	84.7	85.9	84.7	78.6	86.5	89.7	86.5	83.8	68.6	83.5
*MH050915	81.8	83.2	80.3	84.0	82.8	81.7	84.7	82.2	80.7	83.6	75.6	83.3
*MH050917	70.6	94.1	81.2	88.2	90.3	75.6	88.9	96.1	83.8	87.7	68.3	87.4
*MH050916	73.7	89.7	84.7	85.9	84.7	78.6	86.5	89.7	86.5	83.8	68.6	83.5
*MH0509018	73.9	89.9	84.8	86.1	84.8	78.8	86.7	89.9	86.6	84.0	68.8	83.7
*MH050923	77.5	80.9	91.7	81.7	80.2	77.8	82.3	80.1	87.7	82.2	68.5	81.9
*MH050919	79.0	84.7	80.8	80.4	80.5	83.8	81.0	85.0	82.5	78.7	70.2	78.4
*MH050920	76.1	90.1	89.7	86.5	85.1	81.0	87.1	87.5	91.5	83.6	66.8	83.3
*MH050921	79.2	81.9	93.6	83.0	80.8	79.4	83.7	81.4	89.1	82.9	69.1	82.6
*MH050922	79.3	82.0	93.7	83.2	81.0	79.6	83.8	81.5	89.2	83.0	69.3	82.7
*MH050924	79.3	82.0	93.7	83.2	81.0	79.6	83.8	81.5	89.2	83.0	69.3	82.7
*MH050925	76.1	90.1	89.7	86.5	85.1	81.0	87.1	87.5	91.5	83.6	66.8	83.3
HQ264128_TX	73.6	89.5	83.8	92.5	89.8	75.6	93.2	87.0	82.9	97.6	70.5	97.3
EF458275_Arg.	71.7	92.6	82.7	91.0	91.8	77.5	91.6	90.6	87.0	86.9	69.8	86.6
EF458278_Aust.	90.2	75.6	78.0	77.3	76.4	90.2	77.7	75.5	78.6	76.9	68.7	76.6
EF458294_Aust.	100.0	71.0	77.6	72.7	71.5	90.5	73.2	70.7	76.9	74.7	68.0	74.4
HQ264121_Mex.	71.0	100.0	83.3	91.8	89.3	75.7	92.5	95.1	85.1	88.3	70.2	88.0
EF458297_Mex	77.6	83.3	100.0	84.5	82.8	81.4	85.1	82.5	89.9	84.2	67.2	83.9
EF458302_Mex	72.7	91.8	84.5	100.0	92.4	77.1	99.3	90.7	85.7	90.1	70.7	90.1
EF458298_Urug.	71.5	89.3	82.8	92.4	100.0	77.7	92.9	88.2	86.3	89.1	69.3	88.9
EF458299_S Afr.	90.5	75.7	81.4	77.1	77.7	100.0	77.5	75.6	82.8	76.9	68.3	76.6
EF458300_TX	73.2	92.5	85.1	99.3	92.9	77.5	100.0	91.3	86.3	90.8	71.1	90.4
HQ264122_Mex.	70.7	95.1	82.5	90.7	88.2	75.6	91.3	100.0	84.6	87.0	69.2	86.7
KF928960_India	76.9	85.1	89.9	85.7	86.3	82.8	86.3	84.6	100.0	83.6	67.5	83.3
HQ264129_TX	74.7	88.3	84.2	90.1	89.1	76.9	90.8	87.0	83.6	100.0	70.3	99.7
EF458274_Turk	68.0	70.2	67.2	70.7	69.3	68.3	71.1	69.2	67.5	70.3	100.0	70.0
EF458270_Mex	74.4	88.0	83.9	90.1	88.9	76.6	90.4	86.7	83.3	99.7	70.0	100.0

** Similarity Scores (%) **

5. Pairwise identity scores for *Babesia bovis* ITS region among Puerto Rico and GenBank® isolates.

** Identity Scores (%) **

	MH064401	MH064400	MH064417	MH064425	MH064416	MH064421	MH064423	MH064402	MH064414	MH064415	MH064422	MH064418	MH064419	MH064420	MH064424	MH064426	MH064429	MH064430	MH064428	MH064427
MH064401	100.0	100.0	49.1	73.5	49.1	75.8	49.5	73.5	89.4	56.4	56.1	75.3	75.8	75.8	49.1	60.9	88.5	55.0	66.2	60.9
MH064400	100.0	100.0	49.1	73.5	49.1	75.8	49.5	73.5	89.4	56.4	56.1	75.3	75.8	75.8	49.1	60.9	88.5	55.0	66.2	60.9
MH064417	61.8	61.8	100.0	46.2	99.0	47.6	99.5	48.6	52.3	48.0	47.8	47.6	47.6	47.6	98.5	46.0	51.8	49.0	41.2	46.0
MH064425	78.1	78.1	58.5	100.0	46.2	97.6	46.7	94.7	63.5	60.8	58.0	97.1	97.6	97.6	46.2	63.6	62.6	57.1	73.2	63.6
MH064416	61.8	61.8	99.0	58.5	100.0	47.6	99.5	48.6	52.3	48.0	47.8	47.6	47.6	47.6	98.5	46.0	51.8	49.0	41.2	46.0
MH064421	80.4	80.4	59.9	97.6	59.9	100.0	48.1	96.6	65.3	62.2	59.4	99.5	100.0	100.0	47.6	64.6	64.4	58.6	74.5	64.6
MH064423	62.3	62.3	99.5	59.0	99.5	60.4	100.0	49.1	52.7	48.5	48.3	48.1	48.1	48.1	99.0	46.5	52.3	49.5	41.6	46.5
MH064402	79.5	79.5	60.8	96.1	60.8	98.1	61.3	100.0	67.6	63.2	60.4	96.1	96.6	96.6	48.6	65.1	66.2	58.6	75.3	65.1
MH064414	92.2	92.2	62.7	70.8	62.7	72.6	63.2	74.4	100.0	56.8	56.5	65.3	65.3	65.3	52.3	55.0	98.6	56.9	60.6	55.0
MH064415	62.7	62.7	58.9	66.0	58.9	67.5	59.4	68.9	63.6	100.0	94.1	62.2	62.2	62.2	48.0	85.2	55.9	64.5	66.5	85.2
MH064422	61.9	61.9	58.5	63.7	58.5	65.1	59.0	66.5	62.8	95.2	100.0	59.4	59.4	59.4	47.8	83.9	55.6	62.1	64.0	83.9
MH064418	80.4	80.4	59.9	97.6	59.9	100.0	60.4	98.1	72.6	67.5	65.1	100.0	99.5	99.5	47.6	64.1	64.4	58.6	74.0	64.1
MH064419	80.4	80.4	59.9	97.6	59.9	100.0	60.4	98.1	72.6	67.5	65.1	100.0	100.0	100.0	47.6	64.6	64.4	58.6	74.5	64.6
MH064420	80.4	80.4	59.9	97.6	59.9	100.0	60.4	98.1	72.6	67.5	65.1	100.0	100.0	100.0	47.6	64.6	64.4	58.6	74.5	64.6
MH064424	61.8	61.8	98.5	58.5	98.5	59.9	99.0	60.8	62.7	58.9	58.5	59.9	59.9	59.9	100.0	46.0	51.8	49.0	41.2	46.0
MH064426	66.8	66.8	59.4	69.4	59.4	70.3	59.9	71.3	62.7	88.5	87.1	70.3	70.3	70.3	59.4	100.0	54.1	61.1	70.0	98.4
MH064429	91.7	91.7	61.8	70.3	61.8	72.1	62.3	73.5	99.1	63.2	62.3	72.1	72.1	72.1	61.8	62.3	100.0	55.5	59.7	54.1
MH064430	64.2	64.2	62.8	64.8	62.8	66.7	63.3	67.1	66.1	68.5	67.0	66.7	66.7	66.7	62.8	67.5	65.1	100.0	56.3	61.1
MH064428	73.6	73.6	54.9	76.6	54.9	77.9	55.4	79.2	70.1	69.1	66.9	77.9	77.9	77.9	54.9	72.1	69.7	62.8	100.0	70.0
MH064427	66.8	66.8	59.4	69.4	59.4	70.3	59.9	71.3	62.7	88.5	87.1	70.3	70.3	70.3	59.4	98.4	62.3	67.5	72.1	100.0

** Similarity Scores (%) **

6. Pairwise identity scores for *Babesia bovis msa-2b* of the Puerto Rico samples.

** Identity Scores (%) **

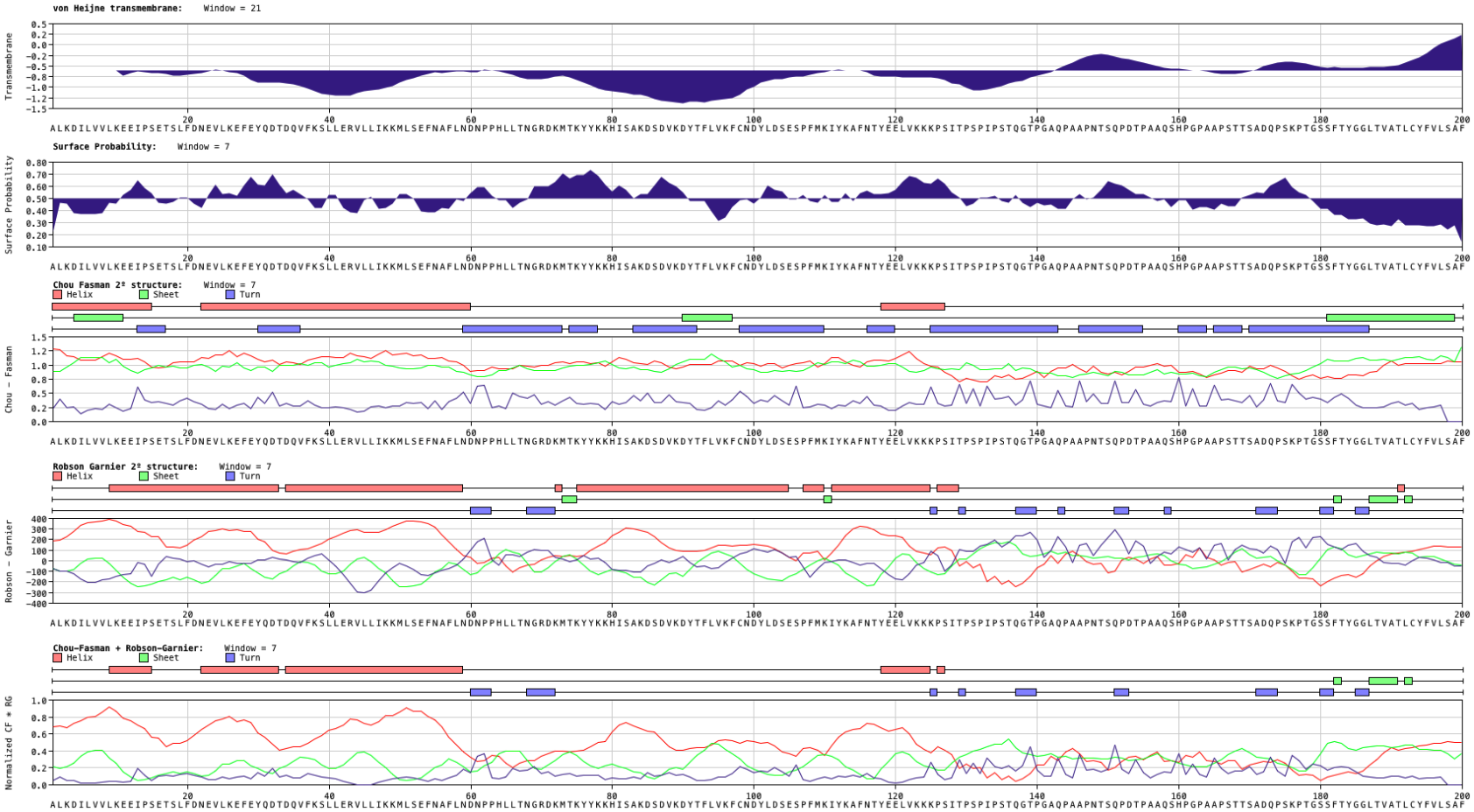
	MH064404_Ames	MH064405_Ames	MH064403_Ames	MH064408_C275	MH064407_C275	MH064409_1986	MH064411_Hust.	MH064410_Hust.	MH064412_C123	MH064413_C123
MH064404_Ames	100.0	45.8	99.6	81.4	81.4	45.4	72.9	56.3	99.2	99.6
MH064405_Ames	59.2	100.0	45.8	58.3	58.3	98.6	59.2	83.1	45.4	45.8
MH064403_Ames	100.0	59.2	100.0	81.8	81.8	45.4	73.3	56.3	99.6	100.0
MH064408_C275	84.3	69.7	84.3	100.0	100.0	57.8	80.3	62.4	81.4	81.8
MH064407_C275	84.3	69.7	84.3	100.0	100.0	57.8	80.3	62.4	81.4	81.8
MH064409_1986	58.8	98.6	58.8	69.3	69.3	100.0	58.7	83.6	45.0	45.4
MH064411_Hust.	79.7	70.0	79.7	87.3	87.3	69.5	100.0	72.3	72.9	73.3
MH064410_Hust.	68.9	87.0	68.9	73.9	73.9	87.4	81.2	100.0	55.9	56.3
MH064412_C123	100.0	59.2	100.0	84.3	84.3	58.8	79.7	68.9	100.0	99.6
MH064413_C123	100.0	59.2	100.0	84.3	84.3	58.8	79.7	68.9	100.0	100.0

** Similarity Scores (%) **

7. Pairwise identity scores for *Babesia bovis msa-2b* of the archived sample.

APPENDIX D

1. Amino acid residues in the epitopes predicted from the deduced amino acid sequences of MH064416 (MSA-2b), were analyzed for their diversity among the Puerto Rico sequences**.**



2. Amino acid residues in the epitopes predicted from the deduced amino acid sequences of *Babesia bovis* (MH064410) (MSA- 2b), were analyzed for their diversity among the culture sample sequences.